Ph.D. Dissertation of Amaël Borzée

Why are anurans threatened?

The case of *Dryophytes suweonensis*

왜 무미양서류는 멸종위기에 처해 있는가?

수원 청개구리의 사례를 통하여

2018년 2월

서울대학교 대학원

생명과학부

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이 논문을 이학박사 학위논문으로 제출함

2017 년 10월

서울대학교 대학원

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Amaël Borzée의 박사학위논문을 인준함

2017 년 12월

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Why are anurans threatened?

The case of *Dryophytes suweonensis*

A Thesis presented by **Amaël Borzée**

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In partial fulfilment of the requirements for the degree of

Doctor of Science, Biological Science

Graduate School of Biological Sciences

Seoul National University

February 2018

Abstract

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The sixth mass extinction is under way, and amphibians are leading the race. An unpredictably high number of amphibian species is under threats of extinction. Factors of population decline in amphibians include (1) habitat destruction, (2) agrochemical and chemical pollution, (3) UV-B radiation, (4) diseases, (5) introduced species, (6) exploitation, (7) climate change and (8) complex causes, being the combined effects of the factors above. For any given species, some factors are of major significance, while other may be considered negligible. The loss of habitat due to urban development and encroachment is a major threat to almost all endangered species. Since the Neolithic Agricultural Revolution, humans have largely settled in lowlands, the primary breeding habitats for the majority of amphibian species. This trend continues nowadays, culminating in metropoles around the world. Encroachment also results in

decreased connectivity between metapopulations, and is linked to the resulting genetic depression. Anthropogenic modification of landscapes also impacts the behaviour and ecology of species, leading to new competition dynamics. For instance, agricultural practices and the loss of natural habitat bring geographically isolated species in contact, potentially leading to introgressive extinction. Finally, the use of chemicals and their presence in the environment, and the introduction of invasive species are factors that can bring a last blow to already weak and destabilised species.

My dissertation aims at assessing the status of the endangered Suweon Treefrog, *Dryophytes suweonensis*, to ultimately predict the species' probability of survival. The species is present on a narrow range on west Korean lowlands, and completes its life cycle in rice paddies. It co-occurs with the widespread Japanese treefrog (*D. japonicus*), with which it interacts on a broad spectrum. The first step of this dissertation was to estimate the entire distribution of *D. suweonensis* in the Republic of Korea. With the data on its distribution and population size, I estimate the meta-population connectivity of the species, and the relationship between range size and fitness depression. I then assess competition, diet overlap and habitat segregation with *D. japonicus*. Because of the spatial arrangement and anthropogenically modified breeding strategy of the two treefrog species in rice paddies, I investigated hybridization between *D. suweonensis* and *D. japonicus* and the possibility of introgressive

extinction in *D. suweonensis*. Besides, I investigated the phylogenetic relation between *D. suweonensis* and other closely related Hylid species in North East Asia to clarify recent evolutionary history. I then assessed the threats linked to the winter ecology of the species, and to chemical run-off resulting from agricultural practices. Finally, I assessed the potential for translocation and reintroduction of the species through a pilot study. I established long term population trends, before running a Population Viability Analysis to assess the probability of extinction for *D. suweonensis*.

This research project is organised under five main parts: (I) demography, (II) behavioural ecology, (III) phylogenetics, and (IV) other factors of population decline and conservation assessment. This dissertation has two intrinsically connected principal aims. First, I determine the threatened status of *D. suweonensis*, in relation to habitat, ecology, competition and disease, and provide possible solutions for its conservation. The second aims is on a broader scale, intending to demonstrate the impact of landscape anthropisation on metapopulation connectivity, exemplify the need for long term surveys to estimate fluctuating population sizes, demonstrate the impact of range restriction on behavioural fitness, retrace evolutionary history through current behavioural patterns, demonstrate the risks of introgressive extinction, highlight the role of resurgence in the Yellow Sea for speciation events,

and finally, demonstrate the relationship between healthy invasive carriers and emergent anuran pathogens. Each of the 14 chapters is linked to the endangered status of *D. suweonensis*, and all highlight a different type of threat that has the potential to accelerate the decline of the species, to the point that it could be extinct within a short period.

Keywords: endangered species, anthropogenic pressure, anuran, *Dryophytes* treefrog, threat, conservation

***Student number*: 2013-31281**

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PART I: DEMOGRAPHY

This part contains all aspects of demographics in relation with the endangered status of amphibians. This includes range description and its limiting factors (chapter 1 & appendix 1), population connectivity and the relationship with population sizes (chapter 2), population trends (chapter 3), description of natural habitat (appendix 2) and a case study around Seoul (appendix 3).

Chapter one

IMPACT OF LAND RECLAMATION AND AGRICULTURAL WATER REGIME ON THE DISTRIBUTION AND CONSERVATION STATUS OF THE ENDANGERED *DRYOPHYTES SUWEONENSIS.*

ABSTRACT

Knowledge about the distribution and habitat preferences of a species is critical for its conservation. The Suweon Treefrog (*Dryophytes suweonensis*) is an endangered species endemic to the Republic of Korea. We conducted surveys from 2014 to 2016 at 890 potentially suitable sites across the entire range of the species in South Korea. We then assessed whether *D. suweonensis* was found in the current and predicted ancestral ranges, reclaimed and protected areas, and how the presence of agricultural floodwater affected its occurrence. Our results describe a 120 km increase in the southernmost known distribution of the species, and the absence of the species at lower latitudes. We then demonstrate a putative constriction on the species ancestral range due to urban encroachment, and provide evidence for a significant increase in its coastal range due to the colonisation of reclaimed land by the species. In addition, we demonstrate that *D. suweonensis* is present in rice fields that are flooded with water originating from rivers as opposed to being present in rice

fields that are irrigated from underground water. Finally, the non-overlap of protected areas and the occurrence of the species shows that only the edge of a single site where *D. suweonensis* occurs is legally protected. Based on our results and the literature, we suggest the design of a site fitting all the ecological requirements of the species, and suggest the use of such sites to prevent further erosion in the range of *D. suweonensis*.

INTRODUCTION

Very few species have a cosmopolitan distribution, and most are likely to be under local environmental pressure (Purvis et al. 2000). When the entire range of a species is threatened by urbanization or other types of habitat modification, the risk of extinction increases exponentially (Huxley 2013). As a result, the assessment of extinction risks depend on threat levels (Mace and Lande 1991; see IUCN 2016), which may guide optimal conservation effort to prevent extinction (Pimm et al. 2014).

Lack of knowledge of species' distributions has already resulted in extinctions that could have been easily avoided. For example, the Tecopa pupfish (*Cyprinodon nevadensis calidae*) became extinct following construction of man-made structures on the Tecopa Hot Springs, the only site where the species occurred (Miller et al. 1989). Unfortunately, this information was not available at the time of

construction. Knowledge of species' habitat preferences provides background information for the assessment of extinction risks (Manne and Pimm 2001), and can be used to develop spatial models for species' distribution (Corsi et al. 2000). For instance, a subspecies of Ursini's viper, *Vipera ursinii graeca*, was known to occur only in Greece and at a single locality in Albania. However, eight new localities were found through landscape and climate modelling, doubling the known range of the species (Mizsei et al. 2016).

Although critical, obtaining information about species' ranges and habitat preferences is only a first step for any conservation effort. At risk species with clearly defined ranges still go extinct in large numbers and a way to stem this loss is through the implementation of protected areas (Pimm et al. 2014). The occurrence of a species within a protected area will significantly increase its chance of survival, despite the debated effectiveness of currently located protected areas (Abellán and Sánchez-Fernández 2015), and the need for the establishment of additional protected areas (Brooks et al. 2004).

The Class Amphibia is currently the most endangered class of vertebrates (Stuart et al. 2004). Among the difficulties for amphibian conservation efforts are unknown distribution limits and the absence of adequate breeding sites. Suitable natural wetlands for amphibians have been converted into farmlands such as rice-paddies over the last century,

especially in the Republic of Korea (Juliano 1993; Czech and Parsons 2002; Machado and Maltchik 2010). Furthermore, those farmlands still holding a fraction of the original biodiversity are being converted into residential and commercial facilities at an alarming rate. In the Republic of Korea, rice production has decreased by about 25 % since peak production in the 1970s (FAO 2016). Since then, there have been clear negative repercussions on habitats available for amphibians (Park et al. 2014).

The Suweon Treefrog, *Dryophytes suweonensis* (previously attributed to *Hyla;* Duellman et al. 2016), is an endangered, endemic treefrog species from the Korean Peninsula. As of 2012, the species was known to occur in a very restricted range, limited to five valleys centred in metropolitan Seoul (Kim et al. 2012b). It is therefore possible that the largest populations of *D. suweonensis* might have been historically present in and around the present Seoul area (Borzée et al. 2015a). Yet, opportunistic observations of calling males in the Democratic People's Republic of Korea (Chun et al. 2012) and further south than previously reported (Borzée et al. 2016c), lead to the expectation of a broader distribution for the species.

Dryophytes suweonensis is an evolutionary important species due to its unusual ZW karyotype, warranting special conservation efforts (Dufresnes et al. 2015). Here, we first aimed to describe the extent of

occurrence and distribution of the species through occurrence surveys, as well as the loss of ancestral range because of urbanisation. We then assessed the overlap between the range of the species and reclaimed tidal flats, and the overlap between range and protected areas. Next, because the distribution of *D. suweonensis* is closely intertwined with rice cultivation, we examined whether the origin of agricultural flood waters was critical for the occurrence of the species. Finally, we extracted environmental variables collected from field surveys and described optimal conservation sites for *D. suweonensis*.

MATERIAL AND METHODS

Field surveys were conducted during 2014, 2015 and 2016, only after the beginning of the breeding season of the species (Roh et al. 2014) to prevent any false negative. Because *Dryophytes suweonensis* has not been observed using other vegetation than rice seedlings as supports from which to hang to produce advertisement calls (Borzée et al. 2016b), and because it is not known to breed in any other wetland than rice paddies (Borzée et al. 2016b, the species typically starts breeding after rice planting.

The setting of modern rice fields during the last decades led to a specific geometric grouping of rice paddies, here referred to as rice-paddy

complexes. A rice-paddy complex is characterized by a central ditch running mostly straight through the complex for irrigation purposes. Along this central ditch, and thus along the longest and straightest line available, usually runs a cemented lane, typically following the centre of the valley. In this study, rice-paddy complexes were considered spatially independent if further than 200 m apart, the maximum daily dispersion distance for the species (Borzée et al. 2016a), or separated by landscape barriers impermeable to treefrogs (Roh et al. 2014).

The Japanese Treefrog, *D. japonicus* is ubiquitously present on the wetlands of the Korean Peninsula, and the two treefrog species are in sympatry at all sites. The advertisement calls of *D. japonicus* and *D. suweonensis* are species specific (Jang et al. 2011; Park et al. 2013), and we noted the presence or absence of *D. suweonensis* through acoustic monitoring. In calling anurans, including Hylids, acoustic monitoring is known to be reliable to estimate population size, and thus adequate to assess occurrence (Weir et al. 2005; Pellet et al. 2007; Dorcas et al. 2009; Petitot et al. 2014; Moreira et al. 2016). In a preliminary study, our aural survey protocol with 5-min transects was accurate to estimate the occurrence of *D. suweonensis* (Borzée et al. 2017c).

Transect surveys

We defined the general area for this study *a priori*, following the ecological requirements of the species such as defined by Roh et al.

(2014) and including all natural and man-made wetlands west of 127.5° E and below 120 m above sea level. This pre-selection of potential breeding sites through Google Earth Pro (Google Earth imagery, v7.1.2.2041, 2013) identified 789 sites in 2014 (Fig. 1.1). A previous study for the occurrence of this species had drawn the southern limit of the range around the Bay of Asan, below 37° N (Kim et al. 2012b; Fig. 1.1). However, our surveys in 2014 demonstrated the southern limit of the range to be inaccurate (Borzée et al. 2016c), and additional surveys were conducted further south in 2015 and 2016, until reaching a point past where the species was no longer detected. In 2015, we surveyed 189 sites, composed of 90 new sites and 99 sites where the species was present in 2014.

A single site where the species was detected in 2014 could not be visited again due to its location within the Civilian Control Zone (CCZ) adjacent to the border with the Democratic Republic of Korea and the lack of permits for 2015 and 2016. In 2016, we surveyed a total of 122 sites (99 sites from 2014, 12 from 2015 and 11 new sites). All accessible sites where the species had been recorded in 2014 were surveyed in 2015 and 2016, even if the species was not detected in 2015. All sites where the species had been detected in 2015 were kept in the list of sites to survey in 2016. In total, 890 sites were surveyed at least once over the three years of surveys.

Surveys were conducted between 5 pm and 2 am, during the peak

calling activity of the species. After arrival at a survey site, five minutes were spent waiting quietly. For each site, aural monitoring was conducted along a single transect along the centre of the rice-paddy complex. A surveyor walked briskly at a maximum speed of *circa* 80 m/min along the transect, noting the presence or absence of *D. suweonensis* at the rice-paddy complex. Before conducting the project, we had empirically measured the detection range for advertisement calls of *D. suweonensis* (n = 20), resulting in a 250 ± 45 m range. The farthest rice paddies in rice-paddy complexes were typically within this detection range.

At the end of each transect survey we recorded water pH and water conductivity (μS) to define the ecological preferences of *D. suweonensis*. We also estimated surface area and longest straight line within sites to determine a sphericity ratio for the occurrence of the species. This is important for determining the likelihood of a species' presence because a circular site will better retain a species than a narrow and linear site. We then recorded the length of continuity with rivers and forests, defined as the continuous line between the edge of rice-paddy complexes and the aforementioned landscape feature, and finally, we noted the presence of buildings and greenhouses within the rice-paddy complexes. These variables were collected through the drawing of polygons or visual inspection of sites in Google Earth Pro (Google Earth imagery, v7.1.2.2041, 2016), at a 10 m resolution, on map dated from

2015 at the latest.

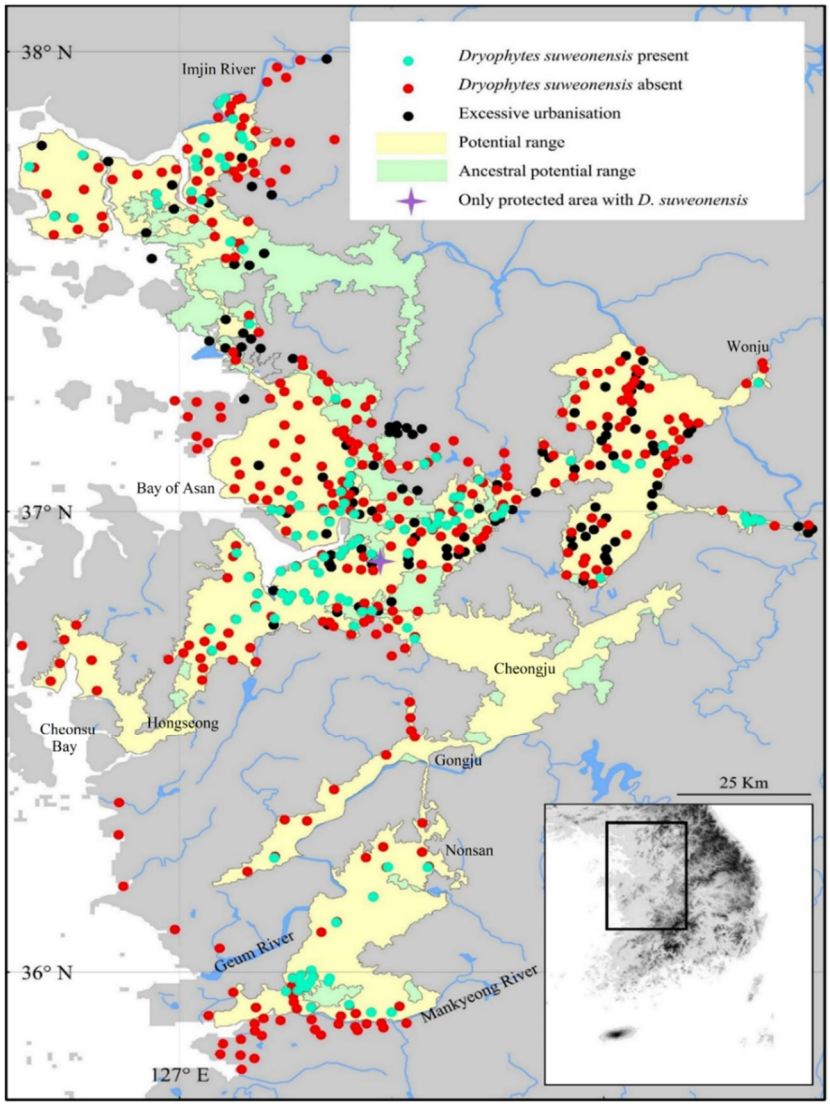

Figure 1.1. Summary of the 890 sites surveyed at least once over the three years of surveys. Dryophytes suweonensis was detected at least once at 114 sites, and 421 sites were too excessively urbanized for the species to occur. Here, potential current range is defined as the range where the species could currently occur, while the potential ancestral range is the range where the species could have occurred before urban development. Pyeongtaek is the area where the only protected area with *D. suweonensis* is found

Reclaimed lands and protected area

To correlate the presence of the species with shifting landscape use, we recorded the presence of the *D. suweonensis* at sites located on reclaimed lands. Here, reclaimed lands used to be mudflats and sea beds, which have been converted into rice-paddy complexes. To record the presence of reclaimed lands, we compared maps from 1950-51 drawn by the US Army (Center of Military History 1990) downloaded in Google Earth and present satellite pictures from Google Earth Pro (Google Earth imagery, 6.2.2.6613, 2016). The 1950-51 maps were selected due to their precision. A land was considered reclaimed if it was not usable for breeding by *D. suweonensis* in 1950-51, but converted into rice paddies before 2016.

We then compared the presence of protected areas and the localities where *D. suweonensis* occurred. Data on protected areas were downloaded from the Protected Planet database, set by the IUCN and UNEP-WCMC (2016). We subsequently noted the number of sites within any protected area, as well as "sites that do not meet the standard definition of a protected area but do achieve conservation in the long-term under national and international agreements" (IUCN and UNEP-WCMC 2016).

Origin of agricultural flood waters

To analyse the impact of agricultural flood water on *D.*

suweonensis distribution, we asked rice farmers for the origin of the water used to flood their rice paddies. This survey was restricted to the general riverine basin surrounding the city of Iksan, south of the Geum River. To be included in the analysis, the origin of the water for a rice paddy complex had to be confirmed by at least two different farmers (Fig. 1.2). Data collection was limited to sites where surveys for *D. suweonensis* were conducted. The area surveyed south of the city of Gunsan and the Mankyeong River had to be excluded from the analysis due to lack of traceability of the origin of agricultural flood water (Fig. 1.2).

Data analysis and optimal conservation sites

For subsequent analyses, we binary encoded the presence of the species, the presence of greenhouses and the presence of permanent human infrastructures within the rice-paddy complexes. We first determined the range of the species, based on presence data points (Fig. 1.1). We defined the potential range of the species based on the non-interruption of landscape variables that are within the range used by the species. We also delineated the ancestral range of the species, defined as the potential range of the species before human development. Namely, a site was considered potential for the species if < 120 m of altitude and within the same water basin as a known population, excluding cities and urban area > 1 km^2 (Fig. 1.1).

We then defined the overlap between species range and reclaimed

area to estimate the land use by the species, and calculated the overlap between species range and protected areas. Descriptive statistics were used to characterise the impact of these landscape variables in both cases.

We hypothesised the origin of the water to be important if linked to the Geum River. This geographic area was chosen due to the clear segregation between areas flooded with water from different origins. We indexed the occurrence of *D. suweonensis* at the sites surveyed in relation to the binary encoding of the origin of flood water, from the Geum River. We subsequently assessed whether distribution of *D. suweonensis* was random in relation to agricultural flood water.

Finally, we developed a plan for an optimal site for the protection of the species. From survey presence data, we calculated averages for water quality (pH and conductivity) as proxies for a larger set of values important for the species (Borzée et al. 2017d), the continuity with rivers and forests, and the sphericity of sites. For sites surveyed over multiple years, the abiotic variables used for the calculation of the species' preferences were restricted to the latest data point. This choice to restrict the analysis to the survey presence data followed recent documented local extinctions, and the potential for other undocumented local extinction due to water quality, salinity, competition and land-use among others, and because these variables are important to ecological preferences of species. All analyses were conducted with SPSS (v. 21.0, SPSS, Inc., Chicago, IL,

USA), and maps were generated with ArcMap 9.3 (Environmental Systems Resource Institute, Redlands, California, USA).

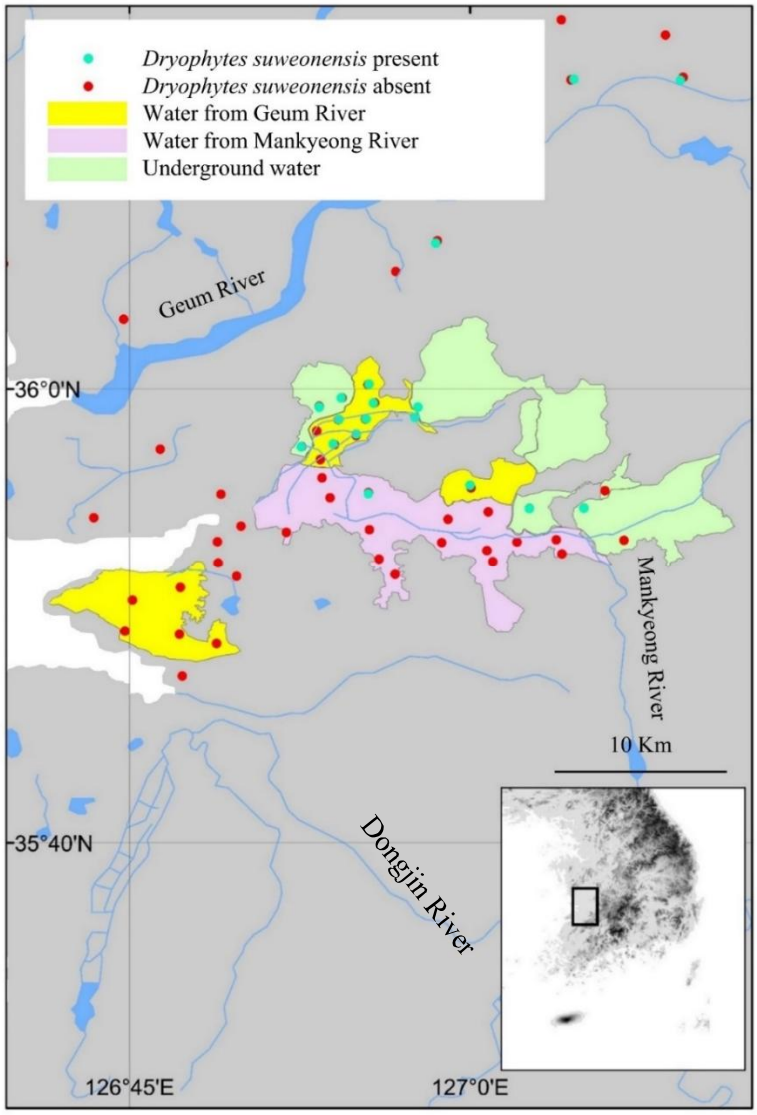

Figure 1.2. Relationship between flood water origin and species presence. Most of the flood water matching with the occurrence of *Dryophytes suweonensis* originated from the Geum River (53.3 %), followed by underground water (40 %), while the remaining 6.7 % of sites were flooded by the Mankyeong river. This analysis is restricted to the area shown on the map.

RESULTS

During the surveys conducted in 2014, only 358 sites out of the 789 sites pre-selected were potentially habitable for the species as urban development and agricultural conversion eliminated the 431 remaining sites. That is, these sites were beyond the ecological requirements of the species as there was no standing water; instead the sites were mostly greenhouses, apartment complexes or dry crops. Within the 358 habitable sites, we found calling *Dryophytes suweonensis* at 100 sites, while the species was not detected at 258 sites. In 2015, calling *D. suweonensis* were detected at 106 sites total, from 94 of the 100 sites where the species was detected in 2014, and 12 of the new sites. In 2016, the species was detected at 109 sites total, 94 of the 2014 sites, 12 of the 2015 sites and three of the new sites. The 94 sites originating from the 2014 dataset where the species was detected in 2015 and 2016 were the same. The species was not detected at the five remaining sites where it had been found in 2014. The 12 sites where the species was detected in 2015 were included in the surveys in 2016, and the species was again detected at all 12 sites. For all subsequent analyses, we assess the species to be present at the 114 sites where the species was detected at least once. This includes the 113 sites surveyed over three years and the site behind the CCZ and these sites are distributed over *circa* 4300 km^2 (Fig. 1.1). However, this species is under significant threats of local extinction at the five sites

where the species was detected in 2014 only; a new motorway was built during the study period in the (Fig. 1.1, Bay of Asan).

Range, ancestral range and current optimal range

The southern boundary of *D. suweonensis'* distribution was extended 120 km southwards from the previous assessment (Kim et al. 2012b). The distribution of *D. suweonensis* ranges from the southern banks of the Imjin River to the northern banks of the Mankyeong River, on a 220 km north-south transect. The range of the species spans 95 km longitudinally, with the westernmost known population in Hongseong area and the easternmost in Wonju (Fig. 1.1).

The potential range of the species, defined as the area where ecological preferences of the species are matched, is situated at the same latitude as the one where the species was detected, but extends 25 km further west from the westernmost site where the species was detected, towards the reclaimed Cheonsu bay. In addition, the corridor of low lands between Nonsan, Gongju and Cheongju matches with the habitat required for the species, but no surveys were conducted in that area, as primarily estimated too far and disconnected from the range of the species to be a potential breeding area. When compared with the potential range of the species before human development, referred here as ancestral range, the land surface area usable by the species decreased by 729 km^2 (Fig. 1.1).

Overlap between reclaimed lands and protected area

Out of the 114 sites where *D. suweonensis* was detected, a total of 30 sites were enlarged and 15 sites were created through land reclamation. The remaining 69 sites were not impacted by land reclamation. When combining all sites impacted by land reclamation, they represent 39.47 % of the sites where *D. suweonensis* was present. When focusing on the overlap between the occurrence of *D. suweonensis* and protected areas, only a single site was selected, South of Pyeongtaek, protected under "Water Source Protection Area". In this protected site, only the riverine system at the edge of the site is protected, putatively used by *D. suweonensis* for hibernation and not for breeding.

Origin of agricultural flood waters

This analysis is based on a subset of sites in the southern distribution of the species (Fig. 1.2). A total of 53.3 % of sites where *D. suweonensis* was present overlapped with agricultural floods originating from the Geum River (Fig. 1.2), highlighting the non-random occurrence of the species in this area. Few sites surveyed in the putatively suitable areas using water from the Mankyeong River had *D. suweonensis* (1/15 sites; 7%), while those in areas using water from the Geum River had higher presence (8/17 sites; 49%) and an even high proportion of sites showed presence in the areas utilising underground water (6/8 sites; 75%). Autocorrelation of the origin of flood water is likely, although of minor

importance in this study and unlikely to impact the result of the statistical analyses.

Assessment of optimal conservation site

The environmental variables for *D. suweonensis* (Table 1.1) showed an average pH of 8.32 and average conductivity of 792.19 μS. The average sphericity was 1.15, meaning that sites were more round than elongated in general. The majority of sites where *D. suweonensis* occurred had permanent man-made infrastructures (52.9 %) and temporary structures (*i.e.* greenhouses, 68.9 %) within the rice-paddy complexes.

Depiction of the sites adequate for the conservation of *D. suweonensis* (Fig. 1.3) was supplemented by vegetation lists from Borzée and Jang (2015), and landscape information matching the current habitat of *D. suweonensis*. Rice paddies are delimited by levees roughly 40 cm wide and 20 to 60 cm high, covered with grasses, and used by treefrogs for basking, foraging, and sheltering (Borzée et al. 2016a). The overhead view of the designed site highlights the need for continuity with forests and rivers to match the preferences of the species (Fig. 1.3a), while the lateral view (Fig. 1.3b) describes depth and vegetation characteristics required for the species.

DISCUSSION

This study highlights the importance of analysing data on the presence/absence and habitat characteristics of species for their conservation. The known range of *Dryophytes suweonensis* was doubled by the data collected over this three years study, highlighting the need for a different approach to the selection of sites for the conservation of the species. These new data show that the increase in known range is due to the inclusion of a large number of sites in reclaimed area from post-war agricultural governmental development. The apparent expansion of the species' known range is, however, countered by several potential local extirpations, such as all the sites in the area of Suweon where the holotype for *D. suweonensis* was described (Kuramoto 1980; Park et al. 2013).

The species still matches the criteria B1ab(i,ii,iii,iv) for listing as "endangered" under the criteria of the International Union for Conservation of nature (IUCN) red list of endangered species. It has an extent of occurrence < 5000 km^2, a severely fragmented population with a continuing observed decline for extent of occurrence, area of occupancy, quality of habitat and the number of locations or subpopulations. At present, the protection of *D. suweonensis* is not ensured because no populations are located within a protected area. Only the edges of a single site are overlapping with a protected area, south of Pyeongtaek. A single

protected site is inadequate for the conservation of an endangered species.

The description of the potential range for *D. suweonensis* shows that an area around Cheongju may be adequate for the species to thrive. However, that area was not included in the initial surveys, due to the lack of knowledge of such a potential wide range for the species. Similarly, sites such as Baengnyeong or Seogmo Islands may be suitable but could not be accessed due to their limited access to non-military personnel. Another potential significant range increase would be within the Democratic People's Republic of Korea, as the species is known to occur around Pyongyang (Chun et al. 2012).

Encroachment on the species' range by development (431 sites), such as at the sites around the city of Suweon, has been partially counter-balanced by the land reclamation projects for rice agriculture (15 sites) implemented at a very large scale in the Republic of Korea during the second half of the last century. The presence of *D. suweonensis* on reclaimed land shows that the species possesses the potential for dispersal despite a lower dispersal ability than the sympatric *Dryophytes japonicus* (Borzée and Jang 2016b). This shift in range is thus linked to rice cultivation and may have been an on-going process since early human agriculture *circa* 5000 years ago (Fuller et al. 2007; Fuller et al. 2008).

Furthermore, numerous *D. suweonensis* populations are isolated

from each other, with urbanization resulting in multiple landscape barriers within and among potential metapopulations.

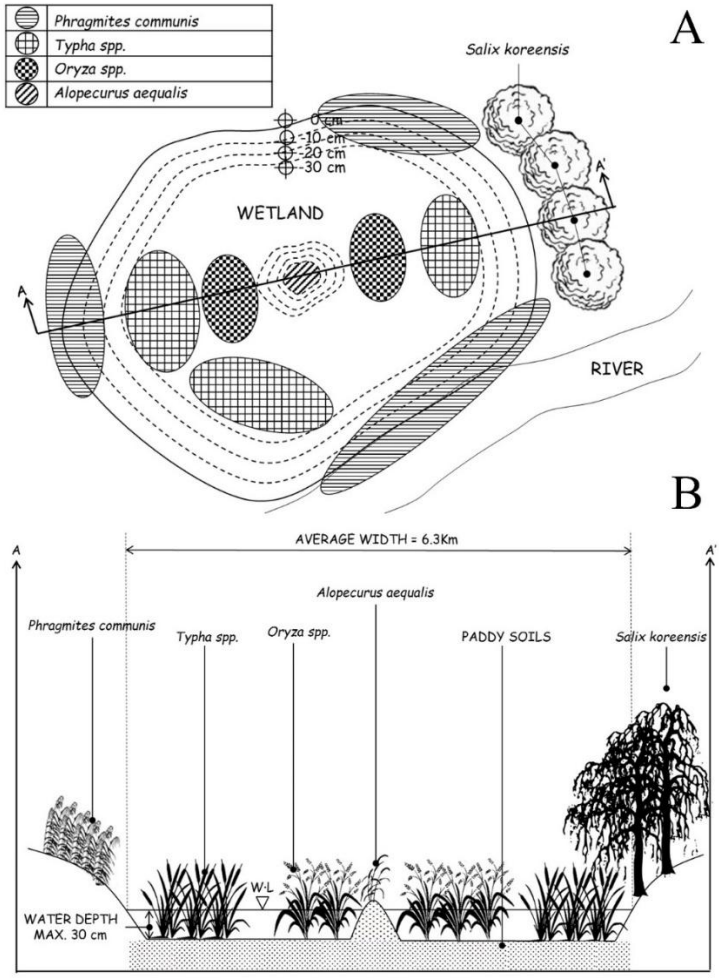

Figure 1.3

(A) Overhead view of the site optimally designed to follow ecological preferences demonstrated by *Dryophytes suweonensis*. The cut AA' is reported in Fig. 3b. The figure is not to scale. Water depths indicated are matching with the average depth of rice paddies, and therefore acceptable if not optimal for the species, and vegetation data is extracted from Borzée and Jang (2015). (B) Lateral view of the site optimally designed to follow ecological preferences demonstrated by *Dryophytes suweonensis*. Water level (WL) originates from the only known natural site with *Dryophytes suweonensis* (Borzée and

Jang 2015).

Table 1.1. Descriptive statistics for abiotic variables of interest collected from all sites where *Dryophytes suweonensis* was present.

	N	Min	Max	Mean	SD
Water pH	114	7.20	10.20	8.32	0.32
Water conductivity (μS)	114	83.50	5720.00	792.19	740.47
Surface area (m^2)	114	0.31	26.09	4.78	4.36
Max. length (km)	114	1.10	301.00	6.30	27.89
Continuity with forests (km)	114	0.00	14.10	3.87	2.83
Continuity with rivers (km)	114	0.00	9.20	1.17	1.79
Sphericity	114	0.01	2.87	1.15	0.65

This calls for a long-term study of population dynamics and network analysis for the species. We would expect the population to be larger at reclaimed sites, due to lower levels of encroachment and fragmentation.

The water origin analysis showed that frogs occur at sites flooded by water originating from the Geum River and underground water sources. However, the species was not detected at sites flooded by water originating from the Mankyeong River. Thus, water originating from underground water bodies and pumped to the surface for agricultural purposes may be adequate for *D. suweonensis*. The areas flooded by river water may be the ones that were seasonally flooded before landscape modifications by humans, and the absence of *D. suweonensis* at some sites could relate to water quality. This idea is potentially supported by the absence of individuals at the only site flooded by water originating from the Geum River south of the Mankyeong River. However, as the water is brought by aerial channels, it is possible that some individuals *D. suweonensis* will drift south to this area in the future and establish new colonies, or perhaps hybridise with the *D. japonicus* present at the site (Borzée et al. 2015b).

Conservation of a species often requires the restoration of the species' habitat (Rannap et al. 2009). The design of an optimal site for the protection of *D. suweonensis* highlights the need for very large continuous

flood plains. However, such large plains are becoming frequently uncommon because of encroachment, and management plans have to be set before these sites disappear. Furthermore, the presence of bullfrogs in the southern part of the range, may have a known negative impact on the species (Borzée et al. 2017c), and demonstrates that the first steps of conservation management plans for the species have to be conducted at any site where the species would be protected/re-introduced. Finally, as the species is still present across a range similar to its ancestral range, we do not recommend ex-situ conservation projects at this time, nor translocations to new sites that would be outside of the ancestral range for the species.

Chapter two

POPULATION CONNECTIVITY AS A REQUIREMENT FOR CONSERVATION EFFORT OF ANURANS.

ABSTRACT

Characterisation, and impact, of landscape elements between populations of the same species, is vital to assess the importance of sites in the frame of population connectivity. *Dryophytes suweonensis* is an endangered species distributed on the lowlands between the yellow sea and the Baekdu Mountain Range on the Korean peninsula. Following population presence assessments, we assessed the connectivity between sites, and populations, and discriminated between connected and disconnected populations. We subsequently assessed the impact of landscape elements on populations and branches. Finally, we estimated the relation between haplotype diversity and population size for a subset of the sites. Our results revealed the isolation of 95 clusters of individuals, themselves distributed into 14 isolated populations, either through distance or barriers. The number of calling males in clusters was significantly related to the number of linking branches < 2 km long. The cumulated populations of clusters connected by branches was significantly related to the presence of roads with four lanes or more, and the cumulated distance of rice paddies making up the length of the branch.

None of the variables recorded was significant towards the population size of populations. Finally, the genetic diversity at a site was significantly explained by landscape factors linked to clusters, such as sphericity. We finally suggest the restauration of connectivity to prevent a drop in fitness for the species.

INTRODUCTION

To be efficient in preserving species and the genetic information they carry, conservation programs need to be implemented at broad a scale (Poiani et al. 2000). Not only the number of individuals surviving is important for conservation, but also the number of populations, the number of individuals for each of these, the effective population size, the connectivity between sites and the potential for gene exchange through individual dispersion (Soulé 1987), as an important but non-exhaustive list. The combination of these factors will be the principal determinant for a species to survive or go to extinction.

A large number of landscape features can have an impact on the connectivity of populations (Forman and Alexander 1998; Geneletti 2004), a principal feature to the survival of species (Fahrig 1997; Dodd Jr 2010). This is especially true for amphibians due to their daily and seasonal displacements (Alford and Richards 1999; Funk et al. 2005;

Cushman 2006; Holderegger and Wagner 2008; Lengagne 2008; Allentoft and O'Brien 2010; Scherer et al. 2012). For instance, two geographically close populations may be separated by a 6-lanes highway, creating a permeable barrier to gene flow (Ashley and Robinson 1996; Trombulak and Frissel 1999). Oppositely, two populations up to ten kilometres apart can still be connected if the landscape feature between the two sites are not preventing dispersion (Smith and Green 2005). Besides, road networks, although crossable individually, can isolate suitable patches of habitat for a species (Bowne and Bowers 2004). Other man-made landscape features such as urban areas wider than 100 m wide (Roh et al. 2014), intensive farming (Forman and Alexander 1998), high speed train tracks (Clauzel et al. 2013) and natural landscape features such as rivers with a breath over 60 m (Angelone and Holderegger 2009; Le Lay et al. 2015) can further increase habitat fragmentation, leading to further genetic partitioning of populations.

The lack of connectivity between populations may results in a loss of genetic diversity, revealed for instance through haplotype networks and comparatively high inbreeding (Allendorf and Luikart 2007; Keyghobadi 2007). Connectivity between populations can be preserved, or improved, through the development of (non-)protected green corridors and ecological buffer zones (Bennett 1998). Landscape connectivity is therefore the result of the interaction between the movement of organisms

and the structure of the landscape (Goodwin 2003), and has functional and structural factors (Brooks 2003; Holderegger and Wagner 2008).

Independently of increased habitat connectivity (i.e. defragmentation, Holderegger and Wagner 2008), focus on the remaining patches of habitat suitable for a species, and their protection and increase in quality, is the first step towards species conservation (Moilanen et al. 2005). The population of a species is analogue to a network in which the survival of each cluster is dependant from the connected clusters, with the whole assemblage of clusters relying on the branches between each of them (Bennett 1998; Moilanen et al. 2005). Therefore the conservation of each branch, although only travelled and not lived in by the species, is important and includes factors such as the preservation of pioneer conditions and the establishment of stepping stone habitats (Tester and Flory 2004; Angelone and Holderegger 2009).

Amphibians are generally not able to disperse over large distances such as would do bird or mammals (Semlitsch 2000), and network connectivity is a primordial factor for the survival of a population. Amphibians are constrained by water balance and thermoregulations, and estimated to be able to disperse/migrate a maximum of 15 km if unaided (Sinsch 1990), with the average typically around 2 km (Sinsch 1990; Sinsch 1992; Miaud et al. 2000; Leskovar and Sinsch 2005; Smith and

Green 2005). Treefrogs, here *Hyla arborea*, have an inherent higher dispersion rate than other ground dwelling amphibians, without however being able to disperse more than *circa* two kilometres per season (Vos et al. 2000; Smith and Green 2005; Arens et al. 2006). For instance, in another study on *H. arborea*, two-thirds of colonisers originated from less than 500 m, a third from 500 to 2500 m and 4 % from ponds up to 5400 m (Le Lay et al. 2015). Also, Angelone and Holderegger (2009) demonstrated that the establishment of stepping-stone habitats lead to migration between populations and the genetic signature of population expansion for a population of *H. arborea*, although previously declining over many years (Weidmann and Flory 1991). Oppositely, a population impacted by a less severe decline, but without habitat network connectivity, displayed the genetic signature of a population with less migrations between populations and a genetically homogenous population. This has an important relation with species survival as the loss of genetic variation is often the result of genetic drift and inbreeding, due to population isolation (Allendorf and Luikart 2007).

To be able to design adequate conservation measures it is first required to collect data on population connectivity and gene flow between each of the populations of the target species. *Dryophytes suweonensis* is an endangered species from the Korean Peninsula (IUCN 2017a; Roh et al. 2014; Borzée et al. 2016c; Borzée et al. 2017b), occurring in sympatry

with the abundant *D. japonicus* on the totality of its range. Several factors may be involved in the decreasing number of individuals for the species, including the lack of natural habitat (Borzée and Jang 2015) and the strong decrease in surrogate breeding habitat (*i.e.* rice paddies; Borzée et al. 2017b).

The purpose of this study was first to assess the connectivity between each population for *D. suweonensis*, through isolation by distance. Second, we aimed at defining population connectivity through the characterisation of natural and artificial landscape elements, thus framing the potential for dispersion between sites. We then correlated landscape variables for a subset of sites with the number of CO1 mtDNA haplotypes to investigate genetic isolation between populations.

MATERIAL AND METHODS.

Species and system description

Dryophytes suweonensis is a small treefrog species solely breeding in rice paddies because of encroachment (Borzée and Jang 2015; Borzée et al. 2017b). It is sympatric with *D. japonicus*, ubiquitously present in wetlands. The advertisement calls of the two species are species specific (Jang et al. 2011; Park et al. 2013), even to the naked ear, and make for reliable signals for field surveys (Roh et al. 2014; Borzée and

Jang 2015; Borzée et al. 2015a; Borzée et al. 2017c). In calling Hylids, acoustic monitoring is reliable to estimate population size (Weir et al. 2005; Pellet et al. 2007; Dorcas et al. 2009; Petitot et al. 2014; Moreira et al. 2016), and our aural survey protocol has been determined to be accurate to estimate occurrence for the species (Borzée et al. 2017c; Borzée et al. 2017b).

The setting of modern rice fields during the last decades lead to a specific geometric grouping of rice paddies, here referred to as rice-paddy complexes. A rice-paddy complex is characterized by a central ditch running mostly straight through the complex for irrigation purposes. Along this central ditch, and thus along the longest and straightest line available, usually runs a cemented lane, typically following the centre of the valley.

Survey protocol

The occurrence of populations used in this study arise from country wide field surveys conducted to determine the presence of the species (Borzée et al. 2017b). Surveys were phenologically situated after rice plantation and after the beginning of diel calling activity to ensure detection and adequate population estimates (Roh et al. 2014; Borzée et al. 2016b) , and encompassed the totality of the species' distribution within South Korea. The protocol used here is described in Borzée et al. (2017b), such as: "After arrival at a survey site, five minutes were spent

waiting quietly. For each site, aural monitoring was conducted along a single transect along the centre of the rice-paddy complex. A surveyor walked briskly at a maximum speed of *circa* 80 m/min along the transect", counting the number of *D. suweonensis* calling at the rice-paddy complex. The detection range for advertisement calls of the species had been empirically measured prior to the surveys, resulting in a 250 ± 45 m range (n = 20). All rice paddies within the rice-paddy complex surveyed were typically within the detection range.

Population occurrence and size estimate

Field surveys for occurrence were conducted during the breeding seasons 2014, 2015 and 2016 (Borzée et al. 2017b). The area surveyed was defined based on *a priori* analyses of potential breeding sites through Google Earth Pro (Google Earth imagery, v7.1.2.2041, 2013), in relation with the ecological requirements of the species (Roh et al. 2014), identifying 890 potential sites, among which 119 were surveyed for population size estimates. Surveys for population estimates were conducted during the breeding seasons 2015, 2016 and 2017, although only 99 clusters were surveyed for the three years. The species was discovered at 19 sites in Iksan (red site on Fig. 2.1) in 2015 only, and thus were surveyed for population size from 2016. One site was surveyed once only in 2015, due to its location within the Civilian Control Zone (CCZ), adjacent to the border with the Democratic Republic of Korea and the lack

of permits to enter the area in 2016 and 2017. The number of individuals at this site was thus not averaged over three years, and was not comparatively different from the population sizes at close sites. The site was not excluded from the dataset due to the known presence of a population, and because species do not respect political barriers.

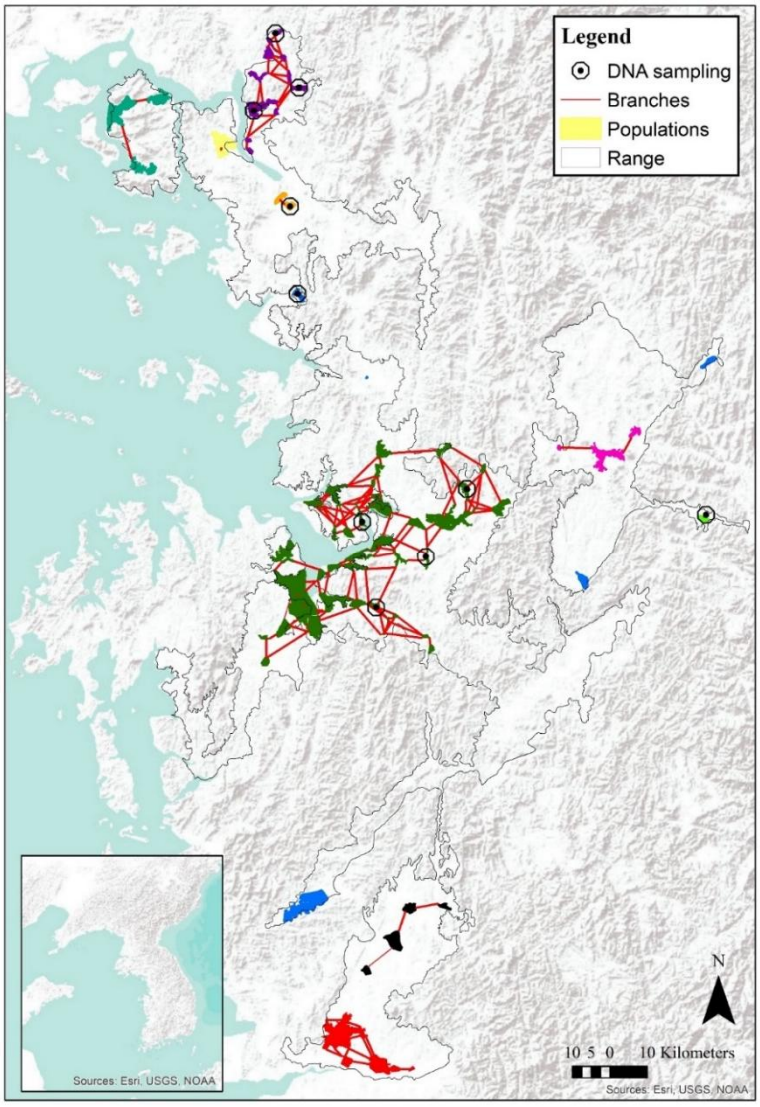

Figure 2.1. Distribution of the 95 clusters of populations. Branches between clusters are in red, and link clusters in 14 populations. In blue are the clusters geographically isolated from others, and the populations are colour-coded such as follow: purple = Paju, orange = Bucheon, yellow = Gimpo, Cyan = Kangwa Island, pink = Icheon, green = Ansan, light green = Chungju, black = Nonsan and red = Iksan. The species range is drawn from Borzée et al. 2017).

Sites were delimited as geographically stopping at the edges of the rice-paddy complexes, and were grouped together in clusters if within daily displacement of an individual (*circa* 200 m), but were not clustered if isolated by distance or landscape barriers. Clusters were grouped into populations when within dispersion distance of individuals, set at 10 km (Pellet and Schmidt 2005).

Landscape data

For each cluster, we also measured landscape variables. We first determined the sphericity ratio for each cluster, calculated such as surface area divided by longest straight line. We did so because a circular site will better retain genetic diversity compared to a narrow and linear site. We then measured the cumulated length of adjacency with rivers and forests, defined as the continuous line between the edge of a rice-paddy complex (*i.e.* cluster) and the aforementioned landscape feature. These variables were collected through the drawing of polygons or visual inspection of sites in Google Earth Pro (Google Earth imagery, v7.1.2.2041, 2016) at a 0.01 km resolution, on map dated from 2016.

When clusters were located within the dispersion capacity of *D. suweonensis*, represented through the dispersion ability of *Hyla arborea*, we considered them linked by branches (Angelone and Holderegger 2009). Branches were drawn if the two closest points of the two focal clusters were 10 km apart or closer (Smith and Green 2005), and not

shadowed by another cluster on the shortest line between the two mentioned clusters. The distance of 10 km was arbitrarily chosen for connectivity following data for *H. arborea* specifying that dispersal over 8 km is rare, while dispersion above 12 km is unlikely (Pellet et al. 2004; Arens et al. 2006; Angelone and Holderegger 2009).

For each branch, we collected additional data: presence of water bodies wider than 60 m (Gibbs 1998; Angelone and Holderegger 2009), presence of urban area wider than 100 m (Ray et al. 2002; Roh et al. 2014) and number of 4-lanes roads, or above (Ashley and Robinson 1996). All these variables are known to be important for dispersion, due to their effect as potential barriers. We then measured the composition of each branch, including the total length from the edge of a cluster to the edge of the next cluster, the total population size of the two clusters connected by the branch, the altitudinal variation between the two clusters, and the potential connectivity between the two clusters linked through continuous rice paddies independently of the sinuosity of the path and distance covered. Finally, we measured the broke-down distance for each branch for the cumulated distances of urban areas, forests, rice paddies and "dry fields". Golf fields and orchards were a significant part of the landscape variables encountered and were assigned to the "dry field" category, due to the presence of chemicals and their linear structure. The presence water bodies were subsequently binary encoded, and the

whole length of branches qualified < or ≥ 2 km for statistical analyses (Smith and Green 2005; Angelone and Holderegger 2009).

Genetic analysis

Genetic sampling was conducted in 2013 and 2014 at non-redundant clusters (permits number 2014-04 and 2014-08), for a total of 87 individuals, originating from ten clusters. Each cluster was separated from the next one by more than 12 km and therefore independent. Each individual was orally swabbed (Broquet et al. 2006), and the swabs were subsequently kept at – 20 °C until DNA extraction. DNA was extracted with the Enzynomic® Genomic DNA Tissue Extraction Kit, following the recommendation of the manufacturer (Genomics Extraction Kit protocol V2013-1). PCR and sequencing for the Cytochrome c oxidase subunit I (mtDNA COI) were conducted following the protocol developed by Jang et al. (2011) based on the primers LepF1 (5'-ACC AAT CAT AAA GAT ATT GGT-3') and LepR1 (5'-CCT CTG GGT GTC CGA AAA ATC A-3'; Hebert et al. 2004). PCR were carried on a PTC-100, BIO-RAD thermocycler (Biorad; USA), with the initial denaturation at 94°C for 3 min, an annealing temperature of 51°C for 35 cycles and a terminal elongation of 72°C for 5 min. Samples were sent to Macrogen Inc. (Seoul, Republic of Korea) for purification and direct sequencing on an ABI PRISM 3100 automatic sequencer (Applied Biosystem Inc., USA).

The sequences were manually trimmed in Geneious (v9.04,

Biomatters Limited, Auckland, New Zealand) Sequences for all individuals and at least 612 bp long were aligned using the Muscle alignment plug-in in Geneious, with a maximum of ten iterations. Sequences were then blasted on the Basic Local Alignment Search Tool from the NCBI portal (http://blast.ncbi. nlm.nih.gov/Blast.cgi) to ensure all individuals were *D. suweonensis*. Haplotype categorisation was conducted with DnaSP (v 5.10 Librado and Rozas 2009), and the number of haplotypes and unique haplotypes were subsequently calculated for each cluster. Due to the low number of individuals sampled in Geumcheon, individuals from clusters 62 and 65 were pooled for the analysis.

Statistical analysis

For ease of redaction, here the number of males producing advertisement calls at a site is summarised by "population size", despite the obvious lack of non-calling females in the count.

Cluster dataset

We analysed the cluster dataset to determine the factors important to population size through a linear stepwise regression. The variables perimeter and surface area were not entered in the analysis and instead the ratio was used. Finally, the total number of branches was also removed from the analysis because it was correlated with the number of branches < 2 km long (Pearson Correlation; $r = 0.76$, $p < 0.001$) and ≥ 2 km long

(Pearson Correlation; $r = 0.28$, $p < 0.001$). In the regression model, population size was set as the dependent variable, and the variables ratio, branch < 2 km and branch ≥ 2 km were set as independent variable. The data were entered through a stepwise backward elimination model. All assumptions were met: (1) the dependent variables was a continuous variables, and there were more than two continuous independent variables. (2) All observation were independent, and without first order linear auto-correlation, such as tested through the Durbin-Watson test (d = 1.52, between the critical values 1.5 < d < 2.5). (3) The visual inspection of scatterplots and partial regression plots demonstrated a linear relationship between the dependent variable and each of the independent variables, and between the dependent variable and the independent variables collectively. (4) The data demonstrated homoscedasticity, visually inspected through the scatterplot of regressed standardised predicted values plotted against the regressed standardised residuals, and because the Skewness and Kurtsosis values were below the standard error values. (5) There was no collinearity of variables, with tolerance values between 0.96 and 0.98, and expected to be > 0.1, and VIF values between 1.02 and 1.05 and expected to be < 10. Besides (6), there was only one outlier, although justified and the violation was ignored, and the residuals were normally distributed.

Branch dataset

The branch dataset was analysed the same way as the cluster dataset. It was also analysed to determine the factors important to the cumulated population size (*i.e.* population size cumulated for the two cluster in contact with the focal branch) through a linear stepwise regression. The variable "cumulated distance" was not entered in the analysis because it was correlated with all other factors (Pearson Correlation; $0.22 < r < 0.67$, $p < 0.003$). In the regression model, population size was set as the dependent variable, and the variables contiguous rice paddies, presence of ≥ four-lane roads, presence ≥ 60 m wide rivers, maximum altitudinal variation and cumulated urban, paddy, forest and dry-field distances were set as independent variable. The data were entered through a stepwise backward elimination model. All assumptions were met: (1) the dependent variables was a continuous variables, and there were more than two continuous independent variables. (2) All observation were independent, and without first order linear auto-correlation, such as tested through the Durbin-Watson test (d = 1.74). (3) The visual inspection of scatterplots and partial regression plots demonstrated a linear relationship between the dependent variable and each of the independent variables, and between the dependent variable and the independent variables collectively. (4) The data demonstrated homoscedasticity, visually inspected through the scatterplot of regressed

standardised predicted values plotted against the regressed standardised residuals, and because the Skewness and Kurtsosis values were below the standard error values. (5) There was no collinearity of variables, with tolerance values between 0.19 and 0.78 and VIF values between 1.28 and 5.08. Besides (6), there no outliers and the residuals were normally distributed.

Population dataset

The population dataset (n = 14) was analysed the same way as the two other datasets. It was analysed to determine the factors important to averaged population size per population through a linear stepwise regression. The variables entered in the analysis were the average ratio per population, the average number of branches < 2 km and the average number of branches ≥ 2 km. The other variables were not entered in the regression model to avoid collinearity. In the regression model, the average population size was set as the dependent variable, and the variables average ratio, average number of branches < 2 km and the average number of branches ≥ 2 km were set as independent variable. The data were entered through a stepwise backward elimination model. All assumptions were met: (1) the dependent variables was a continuous variables, and there were more than two continuous independent variables. (2) All observation were independent, and without first order linear auto-correlation, such as tested through the Durbin-Watson test (d =

1.32). (3) The visual inspection of scatterplots and partial regression plots demonstrated a linear relationship between the dependent variable and each of the independent variables, and between the dependent variable and the independent variables collectively. (4) The data demonstrated homoscedasticity, visually inspected through the scatterplot of regressed standardised predicted values by the regressed standardised residuals, and because the Skewness and Kurtsosis values were below the standard error values. (5) There was no collinearity of variables, with tolerance values between 0.77 and 1.00 and VIF values between 1.00 and 1.29. Besides (6), there no outliers and the residuals were normally distributed.

Genetic analyses

Because a large number of variables were correlated, the importance of the landscape and population variables on the total number of haplotypes and the presence of rare haplotype was tested through a Principal Component Analysis. The variables entered in the PCA for factor reduction were population sizes, number of branches < 2 km, number of branches ≥ 2 km, the total number of branches, perimeter and surface area. The linear relationship between all variables was assessed visually though scatterplots. The PCA was based on eigenvalue greater than 1, with varimax orthogonal rotations, and scores were saved as new variables. The new variables were then set as dependent variables in two ANOVAs, once with the total number of haplotypes, and once with the

number of rare haplotypes, set as factor. All statistical analysis were ran through SPSS v21.0 (SPSS, Inc., Chicago, USA).

RESULTS

Geographic connectivity

The clustering of sites based on daily movement patterns leaded to the isolation of 95 clusters, themselves distributed in 14 populations. Five of the clusters were too isolated to be part of any population, three populations were composed of two clusters, two populations were composed of three clusters and then there was a single population for each of four, 13, 15 and 46 clusters (Fig. 2.1). The largest population was surrounding the bay of Asan (green, 46 clusters), followed by the population around Iksan (red, 16 clusters) and around Paju (purple, 15 clusters). Of smaller sizes, were the populations around Nonsan (black, 4 clusters) south of Icheon (3 clusters, purple) and on Kangwa Island (3 clusters, cyan), followed by the populations of Chungju (light green, 2 clusters) Bucheon (orange, 2 clusters) and Gimpo (yellow, 2 clusters). Finally, 5 clusters (blue) were not included in any population due to their isolation through distance (Wonju, Eumseong, Buyeo and Iksan) or landscape barriers (Sihung; Fig. 2.1).

Cluster dataset

The five clusters with the largest population size had above 100 individuals per site on average over the three years of surveys. Oppositely, there were seven sites with a population of one, or less, in average over the three years of surveys. The average number of individuals at a site was 24.18 ± 13.67 (average ± SD) and the median number of individuals was 30.96. Visual inspection of the dataset highlighted a positive correlation between population size and the number of branches (Pearson correlation; $r = 0.29$, $n = 94$, $p = 0.005$), already highlighting the important factors. All three variables entered in the linear stepwise regression with backward elimination to assess the variables important for the population size were retained. The regression model was a good fit for the data ($F_{(3, 90)} = 3.64$, $p = 0.016$). The prediction of the dependent variable was adequate ($R = 0.33$), while the variance in population size was only explained at 10.8 % by the independent variables ($R^2 = 0.108$). The only variable with a significant model coefficient was the number of branches < 2 km, despite high coefficients values for both the constant and the ratio (Table 2.1). Ad-hoc analysis highlighted an increase in population size with the number of branches < 2 km (Fig. 2.2).

Branch dataset

Before analysis, the landscape variables covered by branches were thought to be important to the combined population size assigned to each

branch. For instance, the presence of ≥ four-lane roads would have a negative impact on the population size, while the presence of rice paddies between clusters would have a positive influence on the population size.

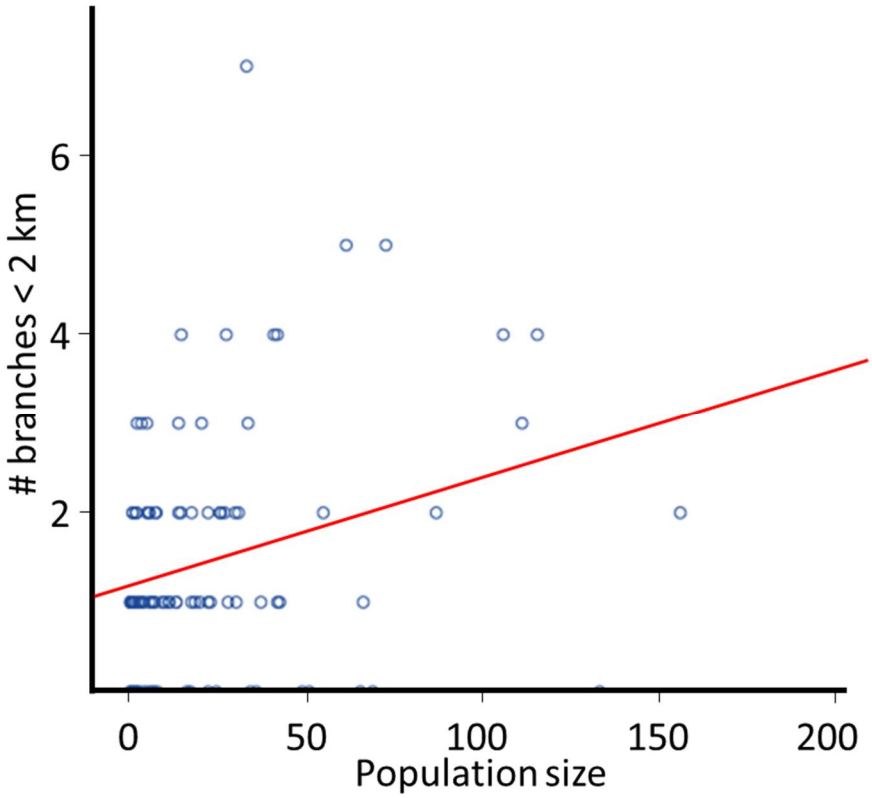

Figure 2.2. Relationship between the population size at a cluster and the number of branches < 2 km connected to the focal cluster. The analysis revealed that the number of branches ≥ 2 km and the ratio surface area / perimeter were not significant. Regression line such as R^2 linear = 0.075; y = 1.18+0.01*x.

Table 2.1. Results of the linear stepwise regression with backward elimination to assess the variables related to clusters important for the population size. All three entered variables were retained, although the number of branches < 2 km was the only variable with a significant model coefficient.

	B	Std. Error	*t*	*p*-value
Constant	5.3	7.877	0.673	0.503
Ratio	7.359	15.642	0.47	0.639
# branches < 2 km	6.838	2.309	2.962	0.004
# branches ≥ 2 km	3.054	1.703	1.793	0.076

The five models of linear stepwise regression with backward elimination were significant, with the *p*-value decreasing for each factor eliminated, from 0.020 to 0.006. However, the proportion of variance in the dependent variable explained by the independent variables did not comparatively vary ($0.10 < R^2 < 0.083$) and the quality of the prediction of the dependent variable went down ($0.32 < R < 0.28$). Besides, the significant coefficients were the same for all models. We consequently selected the model with the best quality of prediction of the dependent variables, and which was a good fit for the data, prior to the first step of backward elimination ($\boldsymbol{F}_{(8, 162)} = 2.35$, $p = 0.020$), thus providing the same results as an entered model. The two variables with a significant model coefficient were ≥ four-lane roads and cumulated paddies, although the maximum altitudinal variation was close to significance (Table 2.2; Fig. 2.3). Ad-hoc plots of the population sizes and all variables showed that as a general rule, *D. suweonensis* was present in larger number when branches were not associated with urban areas, although the extreme encroachment rate blurs the lines and population sizes are more representative of a recent past than the to the current situation (Fig. 2.4).

Population dataset

The five sites with over 100 individuals in average were part of the population around Asan (population 7, green; Fig. 2.1). The next five clusters were from the populations around Asan, Iksan (population 14,

red) and Chungju (population 7, light green; Fig. 2.1).

Among the ten clusters with the lowest population size, four where from the isolated populations (blue populations), one was from the Gimpo population (population 8, orange), two were from the Paju population (population 12, purple) and three from the Iksan population (population 15, red; Fig. 2.1). No clear patterns were visible prior to analysis, although a high ratio value was expected to be important to maintain large populations. However, none of the three models of linear stepwise regression with backward elimination was a good fit for the data. The model with the lowest *p*-value was the one with a single predictor variables: the average number of branches ≥ 2 km ($F_{(3, 10)} = 1.24$, $\boldsymbol{p} = 0.286$). Therefore, we conclude that none of the data collected had a significant impact on the averaged population size per population.

Correlation between landscape specificities and haplotypes

The genetic analysis of the haplotype network displayed a total number of 42 haplotypes, resulting on a haplotype diversity of 0.92. Five of the ten populations studied displayed between five and seven unique haplotype, namely Pyeongtaeck, Wonlong, Asan, Geumcheon and Sihung (clusters 40, 67, 14, 62 and 52). Cheonan and Imjingack (clusters 29 and 72) displayed only two and three unique haplotypes, while the populations in Anseong, Bucheon and Cheongju (clusters 3, 35 and 53) did not display any unique haplotype.

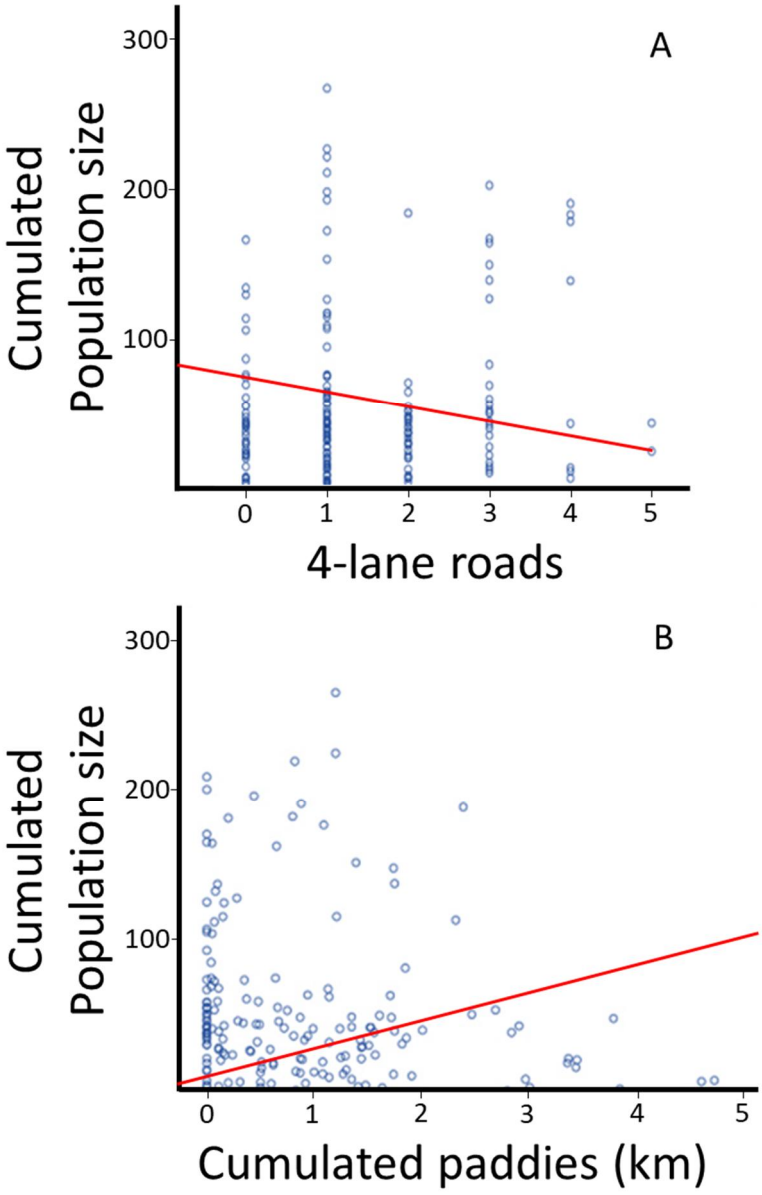

Figure 2.3. Relationship between population size and the number of ≥ four-lane roads (A) and cumulated distance of rice paddies on the straight line between clusters (B). None of the other variables tested were significant.

Table 2.2. Results of the linear stepwise regression with backward elimination to assess the variables related to branches important for the population size. All three entered variables were retained, although the number of branches < 2 km was the only variable with a significant model coefficient.

	B	SE	*t*	*p*-value
Constant	53.77	11.48	4.68	< 0.001
Contiguous rice paddies	7.02	10.06	0.70	0.486
≥ four-lane roads	9.08	4.21	2.16	0.032
≥ 60 m wide rivers	-4.24	10.02	-0.42	0.673
Maximum altitudinal variation	0.32	0.16	1.93	0.055
Cumulated urban	-6.87	5.29	-1.30	0.196
Cumulated paddies	-10.14	4.29	-2.36	0.019
Cumulated forest	-7.80	8.79	-0.89	0.376
Cumulated dry fields	-6.77	5.02	-1.35	0.180

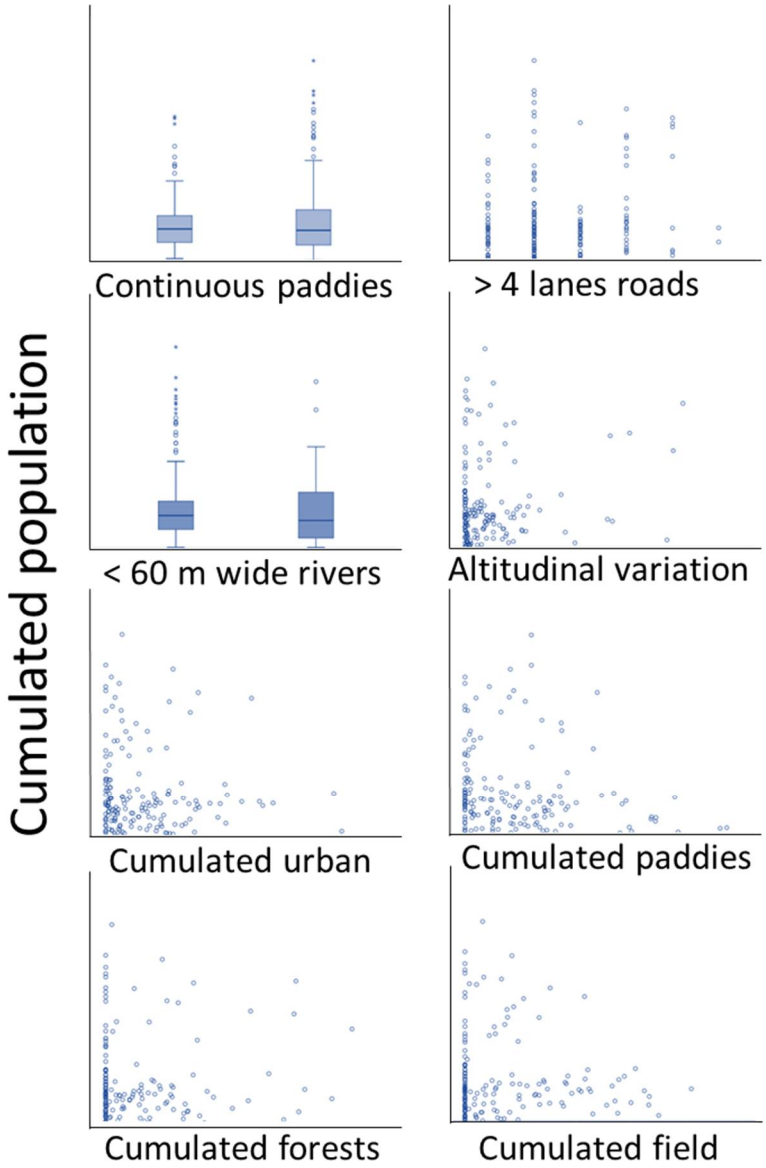

Figure 2.4. Plots for all the variables included in the linear stepwise regression with backward elimination to assess the variables related to branches important for the population size. As a general rule, *Dryophytes suweonensis* is present in larger number when branches are not associated with urban areas, although the extreme encroachment rate blurs the lines of population sizes are more representative of a short past than the data

shown.

The PCA used to reduce the number of factors produced two principal components (PC), together accounting for 81.86 % of the total variance (Table 2.3). The PC1 included all landscapes factors and population size, while PC2 comprised landscape factors related to clusters only.

The subsequent one-way ANOVA for the total number of haplotypes was not significant for PC1 ($F_{(7,2)} = 0.14$, $p = 0.979$), but was significant for PC2 ($F_{(7,2)} = 1.27$, $p = 0.020$). As the factors loading into PC2 were perimeter and surface area, the shape of clusters (i.e. *sphericity*) is important to maintain genetic diversity (Fig. 2.5). The second one-way ANOVA for the presence of rare haplotypes was not significant for PC1 ($F_{(5,4)} = 1.25$, $p = 0.425$), neither than for PC2 ($F_{(5,4)} = 1.39$, $p = 0.385$).

DISCUSSION

The populations of the Suweon Treefrog, *Dryophytes suweonensis*, are distributed in a highly fragmented and urbanised habitat, divided in 15 non-connected populations. The lack of connectivity is partly historical, such as isolation by distance for the easternmost populations, partly induced by the creation of barriers through the anthropisation of the landscape, such as for populations in the vicinity of Seoul. The size of populations in clusters was mostly related to the

connectivity of clusters through branches < 2 km, likely due to the potential for dispersion and gene exchange (Frankham 2005; Albert et al. 2015). The combined populations of two clusters linked through a branch was negatively impacted by landscape barriers such as ≥ four lane roads, and was positively impacted by the cover by rice paddies for the distance between the two clusters. This relationship is likely related to the benefits of connectivity (Biebach and Keller 2009; Jamieson and Lacy 2012).

In amphibians, the lack of connectivity between populations can correlate with the lack of water bodies between populations (Gibbons 2003), and a long circulatory wet dispersion pathway may be more beneficial that a short dry straight line (Popescu and Gibbs 2010). Besides, it is a variable difficult to estimate due to species-specific dispersal abilities (Joly et al. 2003).

We did not find any variable significantly impacting the population size at the population level. The total number of haplotypes was correlated with the number of unique haplotypes, and thus providing and high adaptive potential to populations in Pyeongtaeck, Wonlong, Asan, Geumcheon and Sihung (Fig. 2.1), and the probability of adaptation through evolution (Franklin and Perspective 1980; Lynch 1991; Franklin and Frankham 1998). The relationship with the ratio area/perimeter shows that sphericity is important for genetic diversity but rare alleles are not preserved, and likely lost as a results of encroachment.

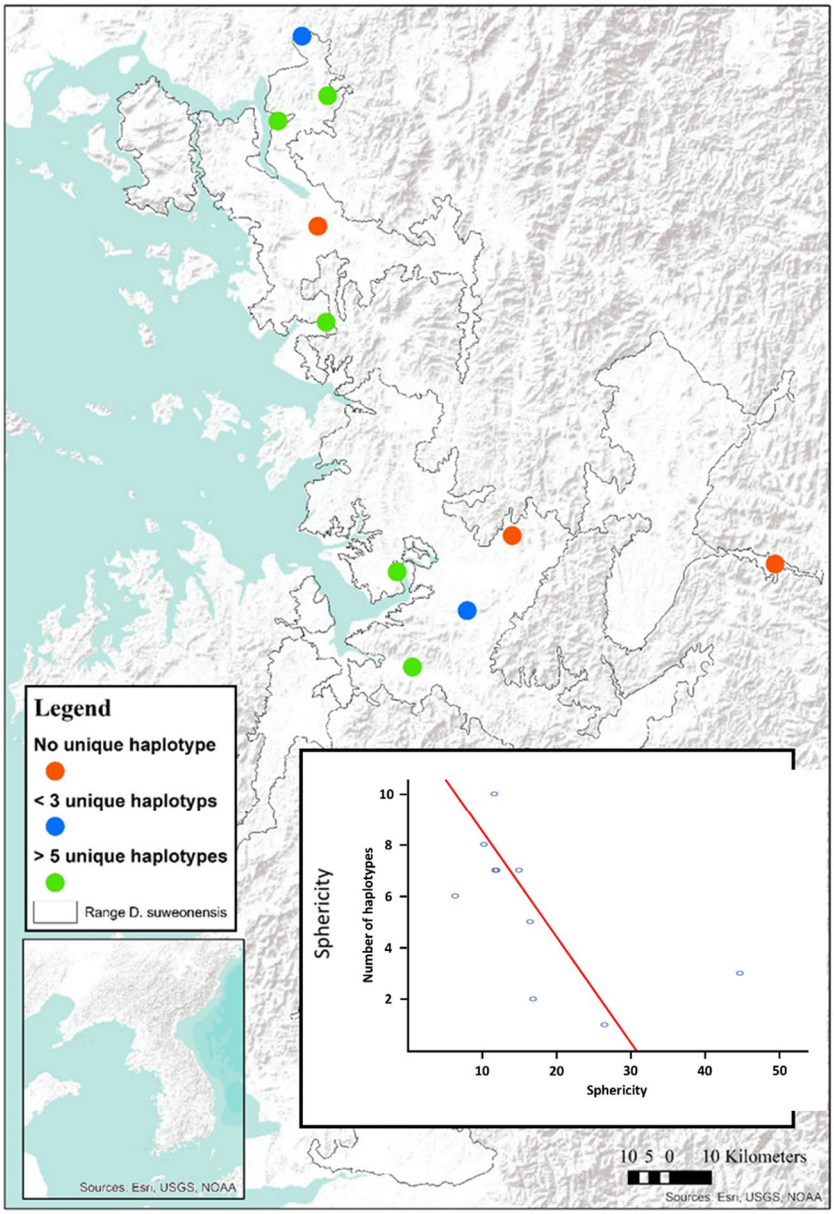

Figure 2.5. Map denoting the geographic location of populations with the different numbers of haplotypes, and inset graph representative of the number of haplotype and the ratio of the two factors loading into PC2: sphericity. This graph highlights an increase in number of haplotypes in relation with a decrease in sphericity.

Table 2.3. Rotated component matrix with loading values for each of the two principal components and Eigen values. The varimax rotation method with Kaiser Normalisation was used. Values in *italics* are the values loading into the corresponding PC.

	Component 1	Component 2
Population size	*0.84*	-0.02
# branch < 2 km	*0.47*	-0.40
# branch ≥ 2 km	*0.97*	-0.10
Total # of branches	*0.94*	-0.23
Perimeter	-0.06	*0.99*
Area	-0.16	*0.95*
Eigen values	3.26	1.24
Percentage of variance	54.42	27.44

Besides, the effects of genetic drift are dependant of the size of the population, and small and isolated populations are more likely to display inbreeding (Frankham et al. 2002; Frankham 2005) and inbreeding depression (Charlesworth and Charlesworth 1997). An allele underrepresented in a population will have a higher probability to go to fixation due to stochastic factors (Ellstrand and Elam 1993), and thus a high population size is important for reduced stochastic effects (Otto and Whitlock 1997) and genetic variation (Lynch and Conery 2003; Crow 2010).

Consequently, to avoid bottleneck at population with low genetic diversity, and not to lose rare haplotypes, we suggest bi-directional translocations. This would result in significant benefits for the long-term survival of the species (Biebach and Keller 2009), such as demonstrated by (Wright et al. 2014). This has already be recommended in similar work, see for instance Jamieson and Lacy (2012).

The long-term survival of a population is based on the functional genetic variation present (Wright et al. 2014), although some bottlenecked species do persist despite low level of MHC variability, such as cheetah (*Acinonyx jubatus;* Castro-Prieto et al. 2011) or southern elephant seals (*Mirounga leonine;* Slade 1992). Thus, field examples and theoretical models have found that evolutionary rescue is possible for species impacted by habitat fragmentation (Pittman et al. 2014), but the definition

of "population at risk" is also linked to a large number of stochastic events that could not be included in the study, such as the presence of pathogens such as the Chytrid fungus (Borzée et al. 2017c) or the sensibility to pollution (Egea-Serrano and Tejedo 2014; Borzée et al. 2017d). Besides, another set of non-predictable factors is the development of urban infrastructures, such as apartment complexes, that can rise in the most pristine environments based on on economical and fashion trends.

An increase in the total number of branches per cluster, through the restauration of sites on the ancestral range of the species (Borzée et al. 2017b) would lead to an increase in the population size. The first positive impact of the creation of green corridors, as stepping stones essential for Hylids dispersion (Angelone and Holderegger 2009), and specifically rice paddies on the Korean Peninsula (Roh et al. 2014; Borzée and Jang 2015), would be a reduction of inbreeding (Bernstein et al. 1985; Michod 1995). It would also influence positively the fitness of individuals on the long term (Lynch 1991): phenotypically, inbreeding is often resulting in a lower fitness for a population due to the expression of recessive or deleterious traits (Chen 1993; Jimenez et al. 1994), which is already the case for *D. suweonensis* (Borzée *Chapter 4*). Consequently, efforts for the creation of green corridors have to be brought forwards for conservation, and especially corridors from and to populations at risk, such as for instance in Sihung (Fig. 2.1; Borzée et al. 2015a). The protection of all sites and the

conservation of the highest genetic diversity possible is important for the species due to the risks linked to genetic drift, inbreeding and low population (Futuyma 1983; Masel 2011; Neher and Shraiman 2011).

The species is endangered (IUCN 2017a) and this study has the potential to designate sites of importance for conservation. However, conservation being a crisis discipline (Chan 2008), preserving all sites where the species is present is unlikely, and a choice has to be made towards isolated sites with rare alleles or sites central to populations, with large population size, but a lower genetic diversity. Here, all clusters non-affiliated with populations were designated as important due to their isolation, as well as all sites once connected to broader populations but now isolated by human activities, such as the ones north of Seoul (Borzée et al. 2017b for historical range). The populations at these sites are most vulnerable, likely to go functionally extinct soon, and therefore require a stronger conservation input. Alternatively, but also additionally, we recommend the preservation of all sites with the largest populations sizes as these belong to large and well-connected populations, and unlikely to have gone trough bottleneck and allele fixation (Borzée et al. 2015b). Finally, we recommend the preservation of key sites for the connectivity of populations. These are first, the population in Paju (purple), important as it is bordering the non-disturbed area of the demilitarised zone between the two Koreas, which hosts several species in a natural setting (Kim

2013), and could possibly connect to populations in North Korea (Chun et al. 2012). The second such site is Sihung, west of Seoul, which is remarkable by the high number of haplotypes presents, and the fact that it is a cluster made of a single site, isolated from any other population, and that it is the only site connecting the populations south and north of Seoul.

Chapter three

POPULATION TREND INFERRED FROM AURAL SURVEYS IN CALLING ANURANS.

ABSTRACT

Amphibian populations fluctuate naturally in size and range and large datasets are required to establish trends in species dynamics. To determine the population dynamics of the endangered Suweon Treefrog (*Dryophytes suweonensis*), we conducted aural surveys in 2015, 2016 and 2017 at each of all 122 sites where this species is known to occur in the Republic of Korea. The focus of this study was to establish population trends rather than population size estimates. We found that the number of calling males was influenced by environmental and landscape variables. Encroachment was also a key factor influencing both the number of calling individuals and the difference in their numbers over the years. While there was a large fluctuation in the number of individuals between years at specific sites, most sites displayed minimal differences in the number of calling males. Finally, when correcting for the overall variation in population size for a given year, we found a decreasing trend over three years, and a significant decrease in the number of calling individuals at certain sites. High rates of encroachment were directly clearly associated with such decrease.

INTRODUCTION

Population decline is widespread in amphibians (Stuart et al. 2004; Pimm et al. 2014), and is attributed to a broad spectrum of causes (Beebee and Griffiths 2005). It is however difficult to ascertain the causes of population decline (Loehle and Weatherford 2017), mainly because of an overall poor detectability of individuals, non-recurrent breeding, breeding at different sites over years, and highly variable populations sizes (Semlitsch et al. 1996; Taylor et al. 2006; Bevelhimer et al. 2008; McCain et al. 2016). Furthermore, some taxa are easier to detect than others and have already been surveyed over long periods (Marsh and Trenham 2008). For instance, non-calling cryptic species such as *Ambistoma cingulatum* are difficult to detect (Bevelhimer et al. 2008), while species producing advertisement calls, such as *Hyla meridionalis*, can be detected more reliably (Petitot et al. 2014). Moreover, species abundance at any site contributes to a higher detection probability compared with species with low population size (MacKenzie 2006; Tanadini and Schmidt 2011). Besides, observers' experience shows a direct impact on the detection probability (Fitzpatrick et al. 2009). Therefore, all amphibian population estimates are subject to biases and inaccuracies, which may not be accounted in models of population dynamics (MacKenzie 2006; MacKenzie et al. 2009)

Long-term monitoring for large numbers of populations enables conservationists to establish trends in population dynamics of focal species. For instance, Petrovan and Schmidt (2016) used data provided by volunteers from Switzerland and the United Kingdom to establish population trends for the common European toad, *Bufo bufo*. Once a trend is established, it can be used to assess the status of a species through the International Union for Conservation of Nature (IUCN) red list (IUCN 2017a), based on both distribution and abundance criteria (see for instance Houlahan et al. 2000 or Cruickshank et al. 2016). Then, the data are used to justify the implementation of conservation management plans (Mace 2014). Therefore, it is critical to establish population trends of endangered species to promote conservation efforts.

Dryophytes suweonensis is a threatened treefrog species endemic to the Korean peninsula (IUCN 2017b ; Borzée et al. 2016a; Borzée et al. 2017a), and restricted to breeding in rice paddies (Borzée and Jang 2015). It is sympatric with *D. japonicus*, ubiquitously present in wetlands, with which it competes for calling space and microhabitat (Borzée et al. 2016b; Borzée et al. 2016c). The range of the species in the Republic of Korea is clearly defined (Borzée et al. 2017a), and the number of sites where it occurs is decreasing because of encroachment. However, no population trend has been established for the species. Because of the difficulties and uncertainty in estimating population sizes, and due to the sampling design

used here, the focus of this study was on a comparative analysis in population trends rather than population size estimates. Here, we surveyed all the sites where *D. suweonensis* was known to occur over three years and related population size fluctuations to environmental factors. We predicted that the population sizes of *D. suweonensis* would not fluctuate drastically as hydrological and environmental conditions surrounding rice paddies remain relatively constant between years.

MATERIAL AND METHODS

The observations conducted here were based on non-invasive call monitoring, and therefore, the laws of the Republic of Korea do not require research permits for such non-invasive surveys.

Species and habitat description

The advertisement calls of *Dryophytes suweonensis* and *D. japonicus* are species-specific (Jang et al. 2011; Park et al. 2013), even to the untrained ear, and are suitable for field surveys (Roh et al. 2014; Borzée et al. 2015; Borzée et al. 2017b). In calling Hylids, acoustic monitoring is used to estimate population size (Weir et al. 2005; Pellet et al. 2007; Dorcas et al. 2009; Petitot et al. 2014; Moreira et al. 2016), and our aural survey protocol has been already been assessed as accurate to estimate occurrence (Borzée et al. 2017b; Borzée et al. 2017a).

The setting of modern rice fields during the last decades led to a specific geometric grouping of rice paddies, which we referred to as rice-paddy complexes. A rice-paddy complex is characterized by a central ditch running mostly straight through the complex for irrigation purposes. Along this central ditch, and thus along the longest and straightest line available, usually runs a cemented lane typically following the centre of the valley.

Survey protocol

The selection of the populations surveyed in this study is based on country-wide field surveys that determined the occurrence of *D. suweonensis* at 122 sites in 2014 (Borzée et al. 2017a), based on data presented by Roh et al. (2014). The surveys were conducted at 116 of the 122 sites where the species occurred during the breeding seasons of 2015, 2016 and 2017. Six sites were not included, due to the need for special permits to access them, unavailable repeatedly over the three years of this study. During each survey we counted the number of calling males as a proxy for population sizes. The surveys were conducted following a protocol robust to variations in occurrence between replicates, although untested for population size estimates (Borzée et al. 2017b). Surveys were timed for maximum detectability of calling males: after rice plantation (Borzée and Jang 2017) and at the beginning of diel calling activity (Roh et al. 2014; Borzée et al. 2016c). They covered the total distribution of the

species within South Korea. The protocol followed the one described by Borzée et al. (2017a): “after arrival at a survey site, five minutes were spent waiting quietly. For each site, aural monitoring was conducted along a single transect along the centre of the rice-paddy complex. A surveyor walked briskly at a maximum speed of *circa* 80 m/min along the transect”, counting the number of *D. suweonensis* calling at the rice-paddy complex. The detection range of advertisement calls of the species was empirically measured prior to the surveys, resulting in a 250 ± 45 m detection range (n = 20). All rice paddies within the rice-paddy complexes surveyed were typically within the detection range.

At the end of each survey, we also recorded the date, time, air temperature (°C), relative humidity (%), air pressure (hPa), light intensity (lx), moon phase (%), water temperature (°C), water pH, and water conductivity (μS). We also estimated scaled variables following the methodology set by Johnson and Batie (2001): wind speed (scale 1-4), cloud cover (scale 1-5), precipitation (scale 1-4), night time cover (scale 1-5). Finally, we recorded the calling index (CI) of *D. japonicus*, *Pelophylax nigromaculatus*, *P. chosenicus*, and the presence-absence of *Lithobates catesbeianus* at the site. The calling index is defined as follows (Mossman et al. 1998): 0- no frog calling, 1- non-overlapping and countable calling individuals, 2- overlapping and countable counting individuals, and 3-overlapping and non-countable individuals.

Statistical analysis

For statistical analyses, the occurrence of *D. suweonensis* was binary encoded for presence-absence. Encroachment was defined as the modification of landscape for at least six contiguous rice paddies within a rice-paddy complex, as a threshold level of two or four contiguous rice paddies lead to encoding all sites as under encroachment pressure. Encroachment was binary encoded for the year when it was detected, but also for the subsequent years as effects are not reversed, even if the extent of the encroachment does not expend. Common examples of encroachment were road construction and building construction, but also included development of power plants, golf fields and quarries, ranked by decreasing frequency. In this analysis, we did not include the conversion of rice paddies to dry agriculture and greenhouses, as these occurred at all sites where the species was present and would have to be quantified over the years to be incorporated in the analysis.

The variables were first verified for correlations to avoid collinearity in subsequent analyses. Because water temperature (Pearson Correlations; $r = 0.83$, $p < 0.001$) and humidity ($r = 0.15$, $p = 0.003$) were correlated with air temperature ($n = 363$), these two variables were not used for subsequent analyses. No other abiotic variables were significantly correlated with each other.

We first analysed the effect of environmental and landscape

variables, such as listed above, with a focus on encroachment, on the calling activity of the species. This analysis is biased in a way that the species was known to be present at all sites surveyed, although the number of calling individuals was different at each site. For this analysis, we selected a Generalised Linear Mixed Model (GLMM) with a linear scale response. The number of calling *D. suweonensis* was the dependent variable, while the predictor variables were divided in two groups: 1) factors: site, date, time of day; and 2) covariates: wind speed, cloud cover, rain intensity, light intensity, air pressure, night cover, moon percentage, water pH, water conductivity, air temperature, CI of *D. japonicus*, *P. nigromaculatus*, *P. chosenicus*, occurrence of *L. catesbeianus*, and encroachment. All the variables were run under a main effect model, with a model based covariance matrix calculated though maximum likelihood estimates.

The model was run under these variables as none of the assumption was violated. That is, all observations were independent, and without first order linear auto-correlation, such as tested through the Durbin-Watson test (d = 1.57, between the critical values $1.5 < d < 2.5$), and there were no outliers. The visual inspection of scatterplots and partial regression plots demonstrated a linear relationship between the dependent variable and each of the independent variables, and between the dependent variable and the independent variables collectively. The data

demonstrated homoscedasticity, visually inspected through the scatterplot of the regressed standardised predicted values plotted against the regressed standardised residuals. Besides, the values of skewness and kurtsosis were below the standard error values. The variables were not collinear as displaying tolerance values between 0.14 and 0.91, and required to be > 0.1 to indicate the evidence of non-collinearity. Besides, the Variance Inflation Factor values were between 1.10 and 7.37, and required to be < 10 to indicate non-collinearity.

Subsequently, we calculated the difference in the number of calling males between 2015 and 2016, between 2016 and 2017, and between 2015 and 2017. A new binary variable was created to represent encroachment for at least one of the three years at each site. We then assessed the difference in the number of calling males between years through Wilcoxon signed tests, as the distribution of the data was strongly skewed. No assumption was violated as there were no extremes outliers, assessed through box-plots visual inspection, although the values for three sites were consistently higher than the others. Finally, the distribution of the paired data set was symmetrical, *i.e.* match in skewness.

We then assessed the impact of encroachment for the three years on the number of calling individuals and the difference between years. Because the dataset was not normally distributed but the residuals were, we used an ANCOVA to fulfil the assumptions. The predictor variables

were continuous and the response variables were categorical, with all observations being independent and no outliers. The Studentised residuals were normally distributed under a Shapiro-Wilk test of normality ($D > 0.79$, $p > 0.05$), and the variances were homogeneous under the Levene's Test of Equality of Variances ($0.97 < F_{(1,117)} < 19.43$, $p > 0.05$). Besides, all covariates were linearly related to the dependent variables, as seen through plot analysis. In the analysis, the six dependent variables were the number of calling individuals for the three years, and the difference in the number of calling individuals between years, while the encroachment variable was a fixed factor, and site was a covariate. The analysis was conducted under a main effect model for all variables.

Finally, we studied the occurrence of local extirpation through descriptive statistics, and spatial visual analysis of heat-maps, representative of changes in the number of calling males based on Kriging interpolations (Fig. 3.1). The Kriging interpolations generate an estimated surface from an unevenly distributed set of points, and the distribution of *D. suweonensis* was used as an inverted cache. To assess the difference in the number of calling males at specific localities, we standardised the impact of variables applying to the whole population by dividing the variable “difference in the number of calling males between two breeding seasons” by the average difference in the number of calling males for all populations for the same breeding seasons, thus creating a ratio. All

statistical analyses were run through SPSS v21.0 (SPSS, Inc., Chicago, USA), and all geographical inferences were run through ArcMap 10.5 (Environmental Systems Resource Institute, Redlands, California, USA).

RESULTS

Relation between number of individuals and environmental variables

The total number of individuals was on average 2510 ± 220.74 over the three years of the study. The number of calling individuals at a site was generally low, and linked to several factors. There were on average 20.74 ± 27.62 (mean ± SD) calling males at a site, $n = 122$ per year, although two sites in 2015 and one site in 2017 were not visited. Thirty-three surveys failed to detect a single calling male, 11 times in 2015, eight times in 2016 and 14 times in 2017, for a total of 19 non-overlapping sites (Fig. 3.1) despite the presence of calling individuals at all sites in 2014. The maximum number of calling individuals at a single site changed between years; it was 109 in 2015, 158 in 2016 and 151 in 2017 (Fig. 3.2).

The results of the analysis to determine the factors important to the number of calling males through a GLMM resulted in a statistically supported model (Omnibus test; $\chi^2 = 1417.95$, $df = 280$, $p < 0.001$) when comparing the fitted model against the intercept-only model. Within the dependent variables, site, date, time of day, wind speed, rain intensity,

light intensity, water conductivity, CI of *P. chosenicus*, occurrence of *L. catesbeianus*, and encroachment were significant (Table 3.1). It is interesting to note that the likelihood ratio for encroachment is fourth, after the factors site, date and time.

Differences in the number of individuals

The difference in the number of calling *D. suweonensis* per site between the years was positive between 2015 and 2016 (3.60 ± 19.26; mean ± SD), with a maximum drop of 45 individuals (1.64 % of 2016 population) and a maximum increase of 112 (4.08 % of 2016 population). The difference in the number of calling *D. suweonensis* per site was however negative between 2016 and 2017 (-2.13 ± 11.37), with a maximum drop of 74 (2.98 % f 2017 population) and a maximum increase of 24 individuals (0.96 % of 2017 population). However, the trend in the number of calling *D. suweonensis* per site over the three years was positive (2017-2015: 1.47 ± 18.16), with a maximum drop of 41 individuals (1.65 % of 2017 population), and a maximum increase of 97 (3.90 % of 2017 population).

The frequencies for population fluctuation were however comparatively higher for minimal differences, with 39.4 % of differences for the range [-2:+2] individuals equal to [-0.07:+ 0.07] % of the averaged population size, and the 10 (= 0.40 %) positive and negative extremes cumulated accounted for 11.2 % of difference (Fig. 3.3).

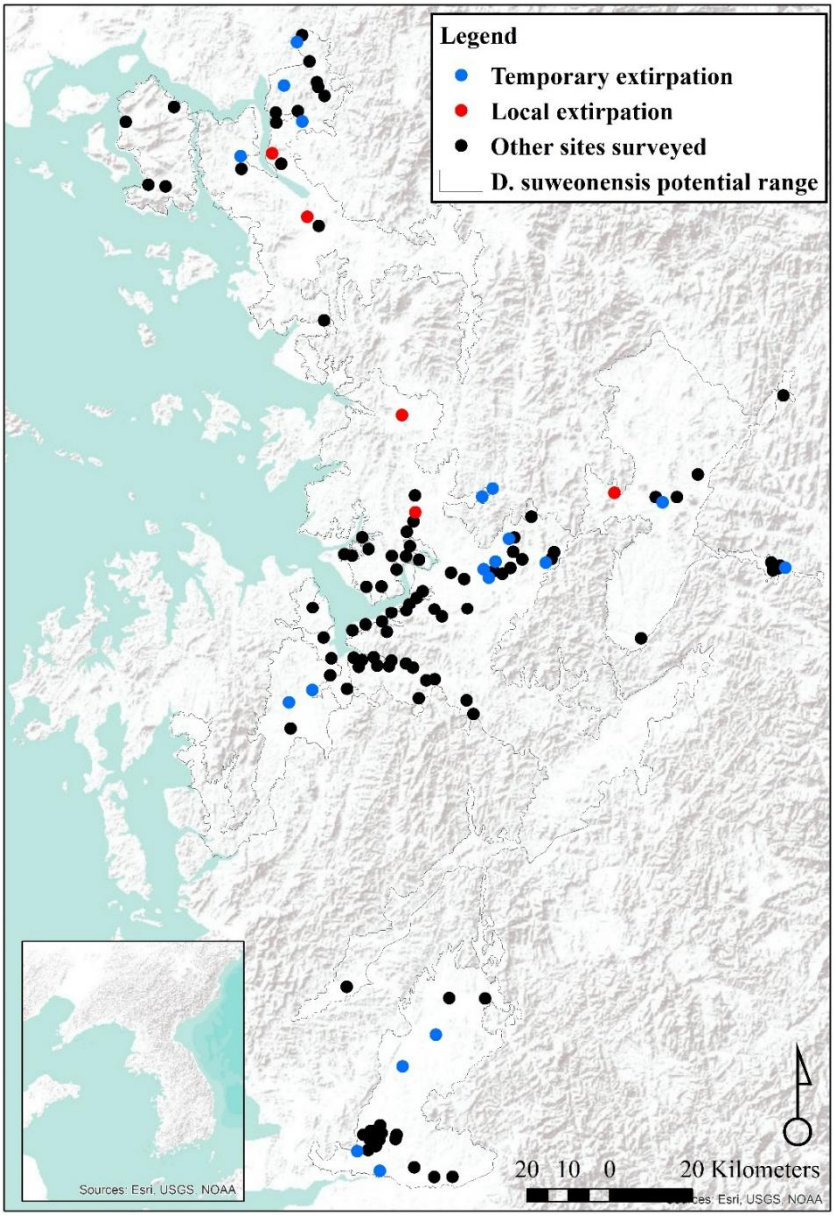

Figure 3.1. Location of the survey sites, local, and temporary extirpations. The species range is drawn from Borzée et al. (in review-a). Map generated through ArcMap 10.5 (Environmental Systems Resource Institute, Redlands, California, USA), with Service

Site was significantly correlated with the number of calling males in 2015 (r = 0.25, p = 0.005), but not in 2016 (r = 0.11, p = 0.239) nor 2017 (r = 0.15, p = 0.102), nor was it significant for any of the three calculated differences in the number of calling males between breeding seasons (0.07 < r < 0.14, p > 0.131). However, site was also correlated with encroachment in 2017 (r = 0.18, p < 0.045), but not for the other years (2015: r = 0.11, p = 0.220; 2016: r = 0.16, p = 0.079). These yearly variations reflect fluctuating dynamics from year to year.

The difference in average number of calling individuals was lower for 2015 (19.20 ± 23.68) and 2017 (20.52 ± 29.64), when compared to that of 2016 (22.48 ± 29.21). When testing for the difference in the average number of calling *D. suweonensis* between the paired years, no significant differences were found between the number of calling males between 2015 and 2016 (Wilcoxon signed tests; Z = -0.86, p = 0.391) and between 2015 and 2017 (Z = -1.25, p = 0.211). Oppositely, a significant difference in the number of calling males was found between 2016 and 2017 (Z = -2.15, p = 0.032). Accordingly, the difference in the number of calling individuals between years was significant for the difference between 2015-2016 and 2015-2017 (Z = -2.23, p = 0.025), while it was not for other periods (2015-2016 and 2016-2017: Z = -0.09, p = 0.925; and 2015-2017 and 2016-2017: Z = -0.66, p = 0.508).

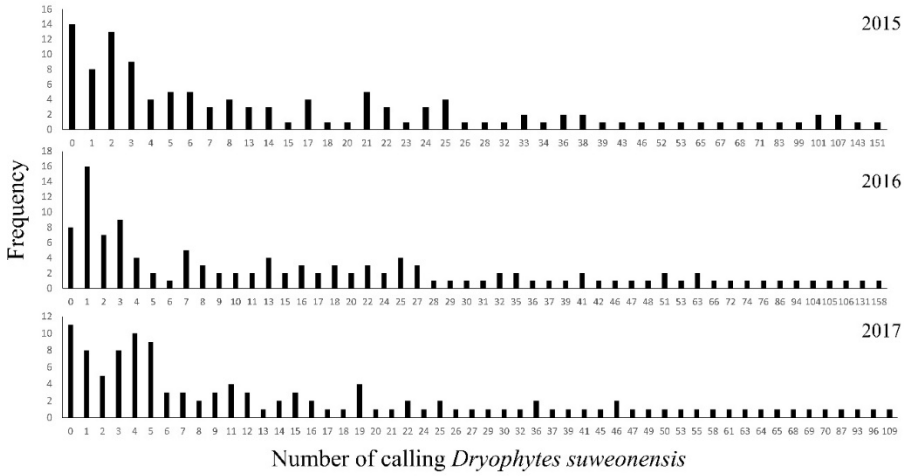

Figure 3.2. Frequency of the number of calling *Dryophytes suweonensis* at the sites surveyed in 2015, 2016 and 2017. The highest number of calling males at a single site was recorded in 2016 and the lowest in 2017.

Table 3.1. Results of the Generalised Linear Model to assess the relationship between the number of calling individuals and abiotic and biotic factors. Significant variables are highlighted in bold.

	Likelihood Ratio χ^2	df	*p*-value
site ID	1129.27	119	**< 0.001**
Date	434.40	52	**< 0.001**
Time	374.38	91	**< 0.001**
Wind speed	4.68	1	**0.030**
Cloud cover	0.48	1	0.489
Rain intensity	7.21	1	**0.007**
Light intensity	6.01	1	**0.014**
Air pressure	2.22	1	0.137
Night cover	3.70	1	0.054
Moon	0.14	1	0.710
Water pH	0.02	1	0.892
Water conductivity	7.31	1	**0.007**
Air temperature	3.37	1	0.066
CI *D. japonicus*	1.15	1	0.283
CI *P. nigromaculatus*	0.31	1	0.577
CI *P. chosenicus*	12.29	1	**< 0.001**
Occurrence *L. catesbeianus*	11.02	1	**0.001**
Encroachment	14.42	1	**< 0.001**

The non-significances of some pairs, despite a higher total number of calling individuals in 2016 (Table 3.2), are due to the lower variations in difference in the number of calling males: 40 for 2015-2017 and 59 for 2015-2016.

Impact of encroachment

Encroachment was found to have a significant negative impact on the number of calling males, as the mean population at site without encroachment was > 24.04 ± 26.87 (n = 363), while it was only > 8.80 ± 14.39 for sites with encroachment during the study period (Table 3.3 and 3.4). The same trend was visible for the difference in the number of calling males between years, although failing to reach significance, with the average for sites with encroachment lower than the one for sites without encroachment for all three years (Tables 3 and 4). The results of the ANCOVA were supported by a significant model (Wilk's Lambda; L = 0.89, $F_{(4.49,3.00)}$ = 114.00, p = 0.005), highlighting a significant negative impact of encroachment on the difference in the number of calling *D. suweonensis* at sites (Table 3.3 and 3.4).

Local extinctions

Compared to confirmed occurrence in 2014, populations went locally extirpated, although sometimes temporarily, at 13 sites in 2015, eight sites in 2016 and 15 sites in 2017.

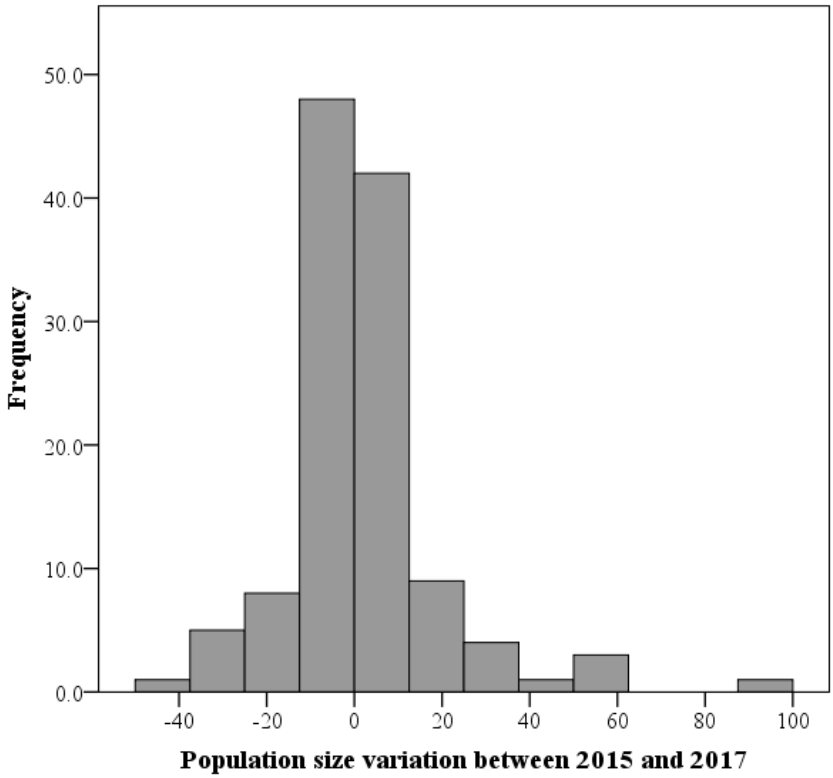

Figure 3.3. Population size variation between 2015 and 2017. Although negative variation reaches – 41 individuals and positive variation reached 94, 90.00 % of the variation is within– 20 and + 20 individuals.

Table 3.2. Descriptive statistics for the variation in the number of calling individuals for the three years paired two-by-two. The highest number of individuals was recorded in 2016, although a lower range of variations was found between 2016 and 2017.

	Mean	Std. Dev.	Min	Max	Range
Difference 2015-2016	3.60	19.06	-45	112	157
Difference 2016-2017	-2.13	11.37	-74	24	98
Difference 2015-2017	1.47	18.17	-41	97	138

A total of 17 sites were devoid of calling individuals for three years in a row, while two sites were free of calling males for two years, and only five sites were deprived of their *D. suweonensis* populations over three years. Out of the 21 sites where extirpation occurred, only six recovered their populations (Fig. 3.1).

When correcting for the general average difference in the number of calling males, the average difference in population size at sites was positive for 2015-2016 (3.78 ± 19.15 = 0.15 ± 0.76 % of averaged population sizes), but negative for 2016-2017 (-2.06 ± 11.41 = 0.08 ± 0.43 % of averaged population sizes) and 2015-2017 (-16.59 ± 27.39 = 0.69 ± 1.14 % of averaged population sizes). This highlights a general negative trend in population size between the first and last year of surveys.

This spatial analysis (Fig. 3.4) is based on the potential range of the species after the removal of urban areas (see Borzée et al., 2017a), and the species is not present in numerous populations on some parts of the range (see Fig. 3.1 for populations). Besides, caution should be taken when reading Fig. 3.4 as the colour gradient is skewed towards a decrease in the number of individuals (dark red), as there were no sites with a sharp increase in number of calling males (green). The non-corrected difference in the number of calling individuals (A, B and C) was geographically consistent for the differences between the two breeding seasons for two localities: on the northern edge of the range (Paju), and at the centre of the

range, around the Bay of Asan (Fig. 3.4). For the maps D, E, and F the values were standardised using a ratio to remove trends at the species' scales and highlight variations at sites. This subsequent analysis also supports the decrease in the number of calling individuals in Paju and around the Bay of Asan, although with some variations in intensity and wideness of patterns. Besides, the patterns in population variations are matching with the non-corrected data, for the respective year-by-year analysis. Interestingly, the patterns for 2015-2017 are almost opposite between corrected and non-corrected data, with the exception of the population decline in Paju and Anseong. It should be noted that the difference in the number of calling individuals does not drastically increase anywhere within the range, while it does go down in large number in some areas..

DISCUSSION

We had hypothesised minimal differences in the number of calling males between seasons because of the relative consistency of agricultural and irrigation practices. This prediction was demonstrated to be relatively correct, as most of the differences were between 2 and -2 individuals (-0.07:+ 0.07 % of averaged population size) per site per season. However, the prediction was wrong in the case of an additional stress factor, here encroachment, as the population dropped for sites where encroachment

was detected. Some populations even went locally extirpated at some of the sites, for non-defined reasons. Overall, we found the number of calling males, as a proxy for population size, to be slowly declining. This trend is however likely linked to the destruction of breeding sites through encroachment. Extirpation seemed to be definitive at the five sites where no individual was detected over the three years of surveys, despite the species being detected in 2014. The loss of these specific sites will lead to the loss of connectivity between the populations south and north of Seoul, and will on the long term result in a loss of genetic exchange, leading to loss of fitness and potential extinction (Soulé 1987; Dodd Jr 2010).

Furthermore, 33 surveys failed to detect a single calling male, at a total of 19 sites. Indeed, large variations in population sizes were expected between years (Semlitsch et al. 1996; Taylor et al. 2006; Bevelhimer et al. 2008; McCain et al. 2016), although usually not leading to the total absence of individuals at sites. This absence is meaningful as large and healthy populations of Hylids are usually characterised by a detection probability > 0.9 (Petitot et al. 2014). Although *D. suweonensis* was present at some of these sites the subsequent year, the populations are in a dire situation, at risk of becoming extirpated (Blaustein et al. 1994), and potentially functionally extinct.

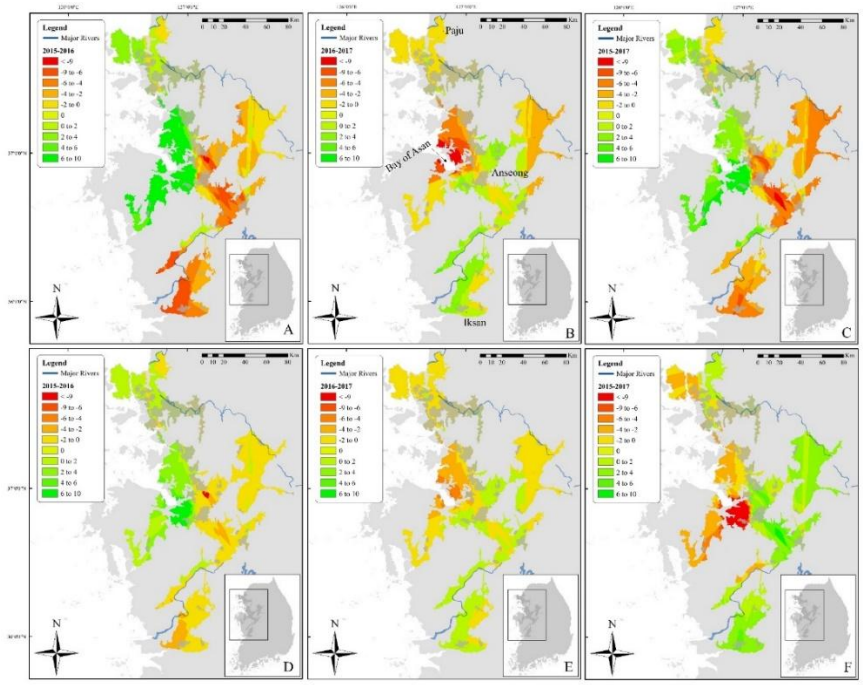

Figure 3.4. Maps representative of differences in the number of calling *Dryophytes suweonensis* between 2015 and 2017. The species range is drawn from Borzée et al. (2017). Maps showing the variation in the number of calling males between (A) 2015 and 2016, (B) 2016 and 2017 and (C) 2015 and 2017. The maps D, E and F follow the same time series as A, B and C, respectively, although based on the corrected differences in the number of calling individuals to reflect differences at sites, independently of the variations at the whole population. Maps based on Kriging interpolations and generated through ArcMap 10.5. Red represents a decrease in the number of calling individuals while green in an increase. Note the unbalanced colour scale.

Table 3.3. Descriptive statistics for the impact of encroachment on the number of calling individuals for each of the three years, as well as for the difference in population size between paired-years.

	No-encroachment		Encroachment	
	Mean	Std. Dev.	Mean	Std. Dev.
Number of calling males 2015	24.04	26.87	10.57	13.65
Number of calling males 2016	28.61	33.17	10.84	15.55
Number of calling males 2017	27.43	34.27	8.80	14.39
Variation 2015-2016	4.57	21.41	0.27	13.25
Variation 2016-2017	-1.19	9.96	-2.05	8.42
Variation 2015-2017	3.39	20.80	-1.77	10.86

Table 3.4. Results of the ANCOVA with the number of calling individuals in 2015, 2016 and 2017, as well as the variation in the number of calling individuals between years set as dependent variable, and encroachment as fixed factor, and the side ID as covariate. Significant variables are highlighted in bold. Δ is used for "variation between".

		Mean Square	*df*	*F*	*p*-value
Encroachment	2015	3765.31	1	7.48	**0.007**
	2016	7952.69	1	10.09	**0.002**
	2017	8377.91	1	10.27	**0.002**
	Δ 2015-2016	773.71	1	2.23	0.138
	Δ 2016-2017	5.54	1	0.06	0.802
	Δ 2015-2017	910.15	1	2.88	0.092
Site ID	2015	3042.16	1	6.04	**0.015**
	2016	423.10	1	0.54	0.465
	2017	1154.20	1	1.41	0.237
	Δ 2015-2016	1196.21	1	3.44	0.066
	Δ 2016-2017	179.67	1	2.04	0.156
	Δ 2015-2017	448.69	1	1.42	0.236
Error	2015	503.27	116		
	2016	788.07	116		
	2017	816.10	116		
	Δ 2015-2016	347.30	116		
	Δ 2016-2017	88.05	116		
	Δ 2015-2017	315.99	116		

The corrected trend over the three years highlighting a slow decline in the total number of *D. suweonensis* individuals (Fig. 3.4 F) is deemed robust and valid, supported by the fact that encroachment was a significant explanatory variable, linked to a decrease in population size. This brings focus on the risks linked to reducing the size of rice-paddy complexes, even if only "a corner" of the site is cut away. This threat is exacerbated by the fact that rice paddies are the only remaining breeding habitat of *D. suweonensis* (Borzée and Jang 2015).

The trend in the number of calling individuals between 2015 and 2017 showed an increase in the number of calling individuals. This number was however a general trend, and was most likely due to discrete environmental variables of the year (Blaustein and Wake 1995). The corrected difference in the number of calling males, comparing the difference in number of individuals to the average difference, shows that there is a decline in population at most north-western sites. These sites are in the most densely populated areas, and are victims of encroachment. The general trend when looking at population sizes is also contradicted by the differences in the numbers of individuals between years, as no significant differences were found in the number of calling males between 2015 and 2016 and between 2015 and 2017.

Finally, the patterns between the map corrected (C) and non-corrected (F) on Fig. 3.4 for the difference in the number of calling males

between 2015 and 2017 were almost opposite. This is due to some outliers sites with comparatively exaggerated changes in population sizes. This result highlights that despite a seemingly general increase in population, local patterns can be hidden, and those provide clues for the slow decline in population size, for instance resulting from encroachment. These differences in the number of calling males are discrete in locality year by year, and insidious, as they are not visible until a large drop in population size at a site. This is typically difficult to detect as aging males, while no longer breeding, are still detected during call surveys. Therefore, once the population decline is detected, conservation plans become much less effective. These discrete minor differences in the number of calling males are thus strongly negatively impacting the whole dynamics of the species on the long term.

The variation in the number of calling males at sites changed with environmental variations (e.g. Salvador and Carrascal 1990; Fukuyama and Kusano 1992; Oseen and Wassersug 2002; Llusia et al. 2013). However, other variables were not important while significance was expected: wind speed (Oseen and Wassersug 2002; Weir et al. 2005), rain intensity (Oseen and Wassersug 2002; Steelman and Dorcas 2010), light intensity (Blair 1961; Navas 1996; Taylor et al. 2007), water conductivity, CI of *P. chosenicus,* (Llusia et al. 2013), and occurrence of *L. catesbeianus* (Borzée et al. 2017b). Thus, the estimated population size

could be modelled from survey data, once corrected for environmental variations. This can be done through the probability of detection of the species, based on its ecological preferences (see for instance Guimarães et al. 2014), although the influence of environmental factors can differ between populations (Berg et al. 2006; Llusia et al. 2013).

Appendix one

SPECIES RANGE: *DRYOPHYTES SUWEONENSIS* (SUWEON TREEFROG).

Dryophytes Suweonensis (Suweon Treefrog). Republic of Korea: North Jeolla: Iksan (35.919927°N, 126.898778°E; WGS 84), 9 m elev. 6 June 2015. Amaël Borzée. Verified by Hyun-Tae Kim. Photographic collection. Natural History Museum Collections, Ewha Womans University (EWNHMPHOTO1, photo voucher). First provincial record extending known range 87 km south (Kim et al. 2012b). Species previously allocated to Hyla (Duellman et al. 2016). Aural surveys conducted at localities where species likely to occur over 120 km S of southernmost previously known locality. A total of 63 calling males recorded.

Appendix two

DESCRIPTION OF A SEMI-NATURAL HABITAT OF THE ENDANGERED SUWON TREEFROG, *DRYOPHYTES SUWEONENSIS.*

ABSTRACT

Amphibian populations have been declining worldwide over the last few decades due to causes like habitat modification, climate change, and emergent diseases. *Dryophytes suweonensis*, a treefrog species endemic to Korea, has disappeared from much of its historic distributional range. Past studies reported this species to breed exclusively in man-made rice paddies. This study reports the habitat characteristics of a semi-natural site for *D. suweonensis*. Out of 100 sites where *D. suweonensis* was present, only one was not a rice paddy. This semi-natural site was characterized by a network of swamps bordered with forests of willow trees. *Dryophytes suweonensis* individuals located on willow trees called from 0.2 to 1.50 m above ground at the edge of the forest near the swamps. The landscape semi-natural site has a large percentage of rice paddies with a very low percentage of urban areas, a feature consistent with findings of a previous study. *Dryophytes suweonensis* was found calling with two other amphibian species. Although extracted from a single site, the information from this study is important for the

management and the conservation of the species.

INTRODUCTION

Anthropogenic events have led to the extirpation of a great number of species (Chivian and Bernstein 2008; Thomas et al. 2004). Amphibians are currently under exacerbated risks of extinction on all continents (Stuart et al. 2004; Wake and Vredenburg 2008). *Dryophytes suweonensis* is a treefrog species resident on the Korean peninsula (Borzée et al. 2013; Borzée et al. 2017b) in sympatry with the abundant *D. japonicus* on its whole range. In a study by Kim et al. (2012b), less than 300 calling males were found, but this number seems to be only a fragment of the whole population. However, the total population size is low enough for the species to have been classified as endangered. Since the description of the species in 1980 by Kuramoto, the species has not been reported breeding in a habitat other than man-made rice paddies (Borzée et al. 2017b). *Dryophytes suweonensis* is therefore characterised as being restricted to anthropologically altered habitats and is consequently dependant on human infrastructures for its survival. As a result, the breeding season of the species coincides with rice cultivation, beginning with water availability in late April and terminating with the drainage of rice paddies in early July.

The breeding habitat of *D. suweonensis* is composed of alluvial plains in the western central Korean peninsula, where rice paddies are in greater proportion than other landscape features (Roh et al. 2014). The two treefrog species in Korea, *D. suweonensis* and *D. japonicus* exhibit microhabitat differentiation for the locations at which they call at night and rest during the day. *Dryophytes suweonensis* calls from the centre of rice paddies, hanging on rice seedlings above water level. Between the periods of mating calls, such as day-time, *D. suweonensis* spends most time on the direct edges of the rice paddies. Conversely, *D. japonicus* calls from the edges of rice paddies and rests, and feeds further away from rice paddies. These characteristics are important for the conservation of *D. suweonensis* as they imply the need for wide and non-disturbed lowland habitats, with a high proportion of vegetation, such as marshes or swamps. Residential and commercial developments have led to the drainage of a large proportion of the range inhabited by *D. suweonensis*. The goal of this study was to describe the semi-natural habitat in which males of *D. suweonensis* were found calling during the breeding season, with an aim focused on conservation.

MATERIAL AND METHODS

Field work was conducted for 90 days starting 16 April 2014 in the provinces of Gyeonggi, Chungcheon, Gangwon and North Jeolla in

the Republic of Korea. We surveyed 480 sites following methods designed through a combination of previously developed amphibian survey protocols (Mossman et al. 1998). Transects were chosen as the best option as Sung et al. 2011 demonstrated that transect surveys are preferable over pitfall and funnel traps, especially for arboreal anurans including treefrogs. All sites adequate for the breeding of *D. suweonensis* were selected through Google Earth imagery (v7.1.2.2041, 2014) over the entire ancestral range of the species (IUCN 2014a). Surveys were conducted between 5 pm and 4 am the next day, covering most of the calling activity of *D. japonicus* (Yoo and Jang 2012) and *D. suweonensis* (Kim et al. 2012b; Kim et al. 2012a). The study period included the breeding seasons of both treefrog species (Roh et al. 2014). Each site was assessed though a single transect on the straightest line available in the rice paddy complex for 5 to 15 min depending on the size of the rice paddy complex. This single transect method appeared to be adequate due to the high rate of detection for calling hylids during the breeding season. For instance, Pellet (2005) detected *H. arborea* 3.3 times for an average of 3.7 surveys.

We noted the presence or absence of calling *D. suweonensis* at all sites. The distinction between *D. suweonensis* and *D. japonicus* was determined following the call properties of the species (Jang et al. 2011; Park et al. 2013). When *D. suweonensis* was detected calling outside rice

paddies, the site was delineated by a concentric circle with a 1 km radius or a continuous area delimited by non-crossable landscape elements, such as 4-lane highways or rivers with a breadth exceeding 100 m (Roh et al. 2014). Information on the position of the calling individuals, and on amphibians and flora species present was collected at the site. Landscape features of the site were also extracted through Google Earth (version 7.1.2.2041; Google Inc.; Mountain View, California, USA).

The land cover around the site of interest was described using descriptive statistics. A Chi-square test was used to determine whether the calling positions of *D. suweonensis* were different between the semi-natural and anthropogenic sites. Habitat type (semi-natural or anthropogenic) and position of the individual calling (water body or edge) were binary encoded for the chi-square test. All statistical analyses were computed through SPSS v. 20.

RESULTS

Dryophytes suweonensis was present at 100 of 480 sites. Only one of these sites was not a cultivated rice paddy. The site was located in Gyeonggi Province, Pyeongtaek, Seotan-myeon, Hwangkuji-ri (37.0760 °N, 126.9985 °E), and was visited on 29 May 2014. This site seemed to have once been used for rice cultivation. However, the site had been

abandoned and had been reclaimed by vegetation. While levees and ditches were still visible, they were breached and did not have the water retention property usually found in rice paddies. The area was divided in two forests of roughly 80 by 160 m, surrounding a swampy area of 90 by 160 m (Fig. A2.1). The forests were characterised by numerous ponds below the trees, from 1 up to 3 m² and 30 cm deep. The depth of the swamps ranged from 18 to 34 cm, with an average of 22.9 cm (SD = 5.41, n = 8). The site was surrounded by similar swamps and forests on a 100-m radius on one of its lengths and rice paddies on the other length (Fig. A2.2). The landscape features calculated from Google Earth showed that the site was situated on an alluvial plain with a width ranging from 0.96 and 1.65 km from the edge of the river to the feet of the surrounding hills, excluding villages. However, the site and the adjacent environment were disconnected from the river and possible flooding or water exchanges by a man-made dam along the eastern side of the river. The single dominant feature of the variables for land cover in the valley, excluding roads and paths, was the rice paddies, which comprised 43.27 % of the area. Abandoned rice paddies represented 40.38 % of the land cover, while green houses and human structures were respectively making up for 14.42 % and 1.92%, of the remaining land cover. The closest human infrastructure, excluding greenhouses, was 470 m away from the site. Forested hills were 780 m away.

Analysis of the species present at the site was conducted for amphibians and plants. The survey for amphibian species revealed three calling species: five *D. suweonensis,* five *Dryophytes japonicus* and three *Pelophylax nigromaculatus*. Netting of the ponds under the trees brought tadpoles of *Rana spp* but no *Hyla* tadpoles, while netting of the swamps did not bring any tadpoles. Out of the 100 sites where *D. suweonensis* was found, calling males were located at the center of the rice paddies in 99 sites (Chi-square test, n = 100, $P < 0.001$).

The lone exception was in the semi-natural site where calling *D. suweonensis* were heard from the edge of the forest towards the swamp, at a tree height of 0.2 to 1.5 m. Separately, the two treefrog species called at higher elevations outside of the rice paddies when in the rice paddy habitats (*D. japonicus*: 153.7 ± 23.2 cm, mean ± SD, $n = 3$; *D. suweonensis*: 82.6 ± 8.6 cm, $n = 4$; Amaël Borzée, personal observation). The vegetation was dominated by five species that differed between the swamp and the forest. In the swamp, 30 cm high foxtail grass, *Alopecurus aequalis*, were prevalent and associated with 1 m high reeds (*Typha spp*). The forest was mainly composed of 5 m high deciduous Korean willows, *Salix koreensis,* and occasionally 1.5 m high grasses (*Elymus repens and Phragmites communis*).

DISCUSSION

Since its description in 1980 (Kuramoto 1984), *Dryophytes suweonensis* was known only from rice paddies. In this study we report a semi-natural habitat of this species for the first time.

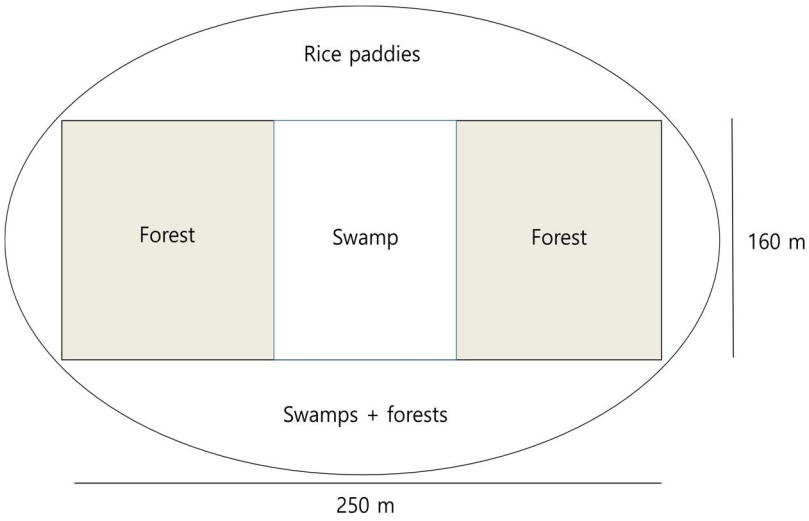

Figure A2.1. Schematic representation of the semi-natural site for *D. suweonensis*. The individual was heard calling on the edge between swamp and forest on the right side of the schematic representation.

Figure A2.2. Picture of the semi-natural breeding site for *D. suweonensis* showing the swampy area on the foreground and the forest on the background. Males were found calling at the edge between these two habitats.

The areas surrounding the semi-natural habitat of *D. suweonensis* in this study largely fit into the geographical requirement of *D. suweonensis* habitat based on a Geographical Information System analysis in which the sites harbouring *D. suweonensis* were dominated by rice paddies with minimal human infrastructure (Roh et al. 2014). The semi-natural habitat is located at the centre of the distributional range of *D. suweonensis* and is close to the sites with the highest number of *D. suweonensis* individuals found to date (Kim et al. 2012b; Amaël Borzée, personal observation). Thus, because of the high dispersal probability, the populations found in the semi-natural site and the surrounding area are the least likely to become extinct through the stochastic process of local extinction and recolonisation (Marsh 2001).

The striking contrast between the semi-natural site and rice paddies is the surface of water bodies. During most of the breeding season, the surface of rice paddies is open with low density of vegetation. Although the vegetation density in rice paddies increases by July and August, the breeding season of *D. suweonensis* is restricted at that time of the year due to the drainage of rice-paddies. In contrast, the semi-natural site is heterogeneous with numerous small ponds scattered in dense vegetation. The heterogeneous landscape of the semi-natural site may replicate the natural habitat before the development of rice cultivation between 10 000 and 5 000 years ago (Liu et al. 2007; Fuller et al. 2007;

Fuller et al. 2008). Oppositely, a similarity between the semi-natural site and rice paddies is the depth of approximately 0.20 m. The shallow water depth may be the reason why rice paddies are an appropriate surrogate for breeding in several amphibian species, including *D. suweonensis* (Jang and Borzée 2014).

The position of all hylids on the edge of the willow forests at this semi-natural site suggests that breeding is possible either in the swamp or in the water bodies in the forested area. The spatial location of the two hylid species diverged from the situation in rice paddies as both species were heard calling from the edge of the forest, and not from the swamp. The presence of *D. japonicus* at the breeding site may prevent *D. suweonensis* from establishing calling positions at the edge of the rice paddies, and males of *D. suweonensis* might be displaced towards the centre of rice paddies. When *D. suweonensis* is the only hylid species at the site, or with a low number of *D. japonicus* individuals, males may be able to establish calling position on trees at the edge of the wetland. However, this hypothesis is difficult to test due to the low population size and occurrence of *D. suweonensis*, in opposition to the abundant *D. japonicus*. Also, because all hylids were heard calling from higher than the level at which they called in rice paddies, it raises questions concerning the microhabitat choice shown by these species outside of the rice paddy system. Agricultural development may have resulted in the loss

of species-specific stratification in *D. japonicus* and *D. suweonensis* in Korea (see Gondim et al. 2013).

Our observations are important for conservation purposes as *D. suweonensis* was, so far, not known to breed outside of the rice paddies. For example, the vegetation structure and landscape features in the study site may be used to infer a possible restored habitat for *D. suweonensis*. *Dryophytes suweonensis* is strongly dependant on rice paddies for breeding, and any alteration of the agricultural system, such as transformation of rice paddies into green houses or use of the land for various other purposes, could be fatal to the species. The semi-natural site where *D. suweonensis* was found calling indicates that either re-colonisation is possible or that a site with rice paddies left unattended can still host the species. The results can be used as an example of a possible setting for the conservation of *D. suweonensis* or its reintroduction.

Appendix three

SEOUL, KEEP YOUR PADDIES! IMPLICATIONS FOR THE CONSERVATION OF HYLID SPECIES.

ABSTRACT

Biodiversity is plummeting worldwide, and the major causes of such decline include habitat degradation and climate change. While cities do contribute to the negative impact to the environment, they can also serve as strategic centres for conservation programs. Sites qualifying as biogeographic islands within metropolitan Seoul were studied for the occurrence of two hylid species: the endangered *Dryophytes suweonensis* and the abundant *D. japonicus*. This study demonstrates that neither habitat diversity nor surface area, but solely the occurrence of aggregated rice paddies is a requisite for *D. suweonensis*, hypothetically due to its strict breeding requirements. On the contrary, *D. japonicus* occurrence was not affected by any of these factors, and all types of habitats studied were adequate for this species. The presence of an endangered species within the boundaries of one of the most populated metropolises suggests a strong natural resilience, which should be enhanced with appropriate actions. We emphasize that the management plans therein can, and should, be used as the first step in the conservation of *D. suweonensis* in metropolitan Seoul.

INTRODUCTION

Resulting from factors such as climate change and anthropogenic habitat modification worldwide, biological diversity is decreasing at such a speed that it has been termed the "sixth mass extinction" (Wake and Vredenburg 2008; Wake 2012). Accordingly, the current rate of extinction is up to 10,000 times faster than the background rate inferred from fossil record (Singh 2002). Up to 30 % of the species currently known are predicted to have disappeared by 2050 (Thomas et al. 2004), subsequently reaching 50 % by the end of the century (Singh 2002). High extinction rates have led to the extensive study of extinction processes for many taxa (e.g., Castelletta et al. 2000), although conservation schemes are still too uncommon and the number of endangered species is constantly increasing (Brown et al. 1998).

Cities and their suburbs are notorious for their negative impact on the environment. Early human settlers were attracted to areas rich in natural resources, which later grew into cities. However, the same areas are equally sought after by other species and are becoming less accessible as cities are expanding (Olson and James 1982; Steadman 1995; Myers et al. 2000). For instance, the flood plains where metropolitan Seoul now stands, with roughly 20 million inhabitants, used to be covered by wetlands (Won 1981), and therefore acted as

biodiversity reservoirs (Contini and Cannicci 2002). When wetlands were drained and built upon, the biodiversity present at the site disappeared through co-extinction, and only biogeographic islands remained. Some exceptions persisted through substitute habitats in the form of rice paddies. These were soon replaced by other human-dominated structures, which slowly pushed species away from their original living sites. This situation is recurrent worldwide, under different modalities, and has brought wetland organisms to the front of the extinction queue (Abell 2002), in a strongly unbalanced situation that favours terrestrial organisms over their freshwater counterparts (McAllister et al. 1997). Consequently, amphibians have been the subject of severe population declines over the last several decades, with approximately a third of all species under threat of extinction (Wake 2012), while more than a hundred have already gone extinct (Stuart et al. 2004).

A biogeographic island is similar to a literal island in that it is surrounded by unsuitable habitats, such as urban tracts, that prevent the immediate dispersion of species (Simon 2008). The city of Seoul is surrounded by a green belt, which is a series of forests and rice fields encompassing the city (Bae 1998). Residential and commercial developments are typically prohibited in most areas of the green belt. However, this green belt does not amount to a continuous habitat, but to a fragmented continuation of ecologically dissimilar habitats. These are

biogeographic islands formed by the complex and abundant urban tracts separating them. The aim of this study was to define the habitat characteristics of the endangered *Dryophytes suweonensis* (Ministry of Environment of the Republic of Korea 2012; IUCN 2017a), in relation to the abundant *D. japonicus* within metropolitan Seoul. The landscape habitat preferences described for the species (Roh et al. 2014; Borzée and Jang 2015) were used to discuss the habitat characteristics and then suggest conservation strategies.

MATERIALS AND METHODS

Biogeographic islands within, or partially included within, the cadastral area of the city of Seoul were identified and characterised for their surface area and distance to the closest island from Google Earth (v7.1.2.2041, 2014; Google, Mountain View, CA, USA). In case an island was only partially within the boundaries of Seoul, we extended the analysis to its contiguous entirety. We also annotated the habitat type from the publicly available database of Daum maps (v 3.9.12; Daum Communication, Seoul, Korea) dated from 2011 for each island, and no inconsistency were noted with the data from 2014. The limits between geographic islands were defined through landscape barriers that made dispersal unlikely, i.e., physical obstacles that greatly increase mortality risks for most amphibian species (Ray et al. 2002; Roh et al. 2014). We

considered roads with four-lanes or wider (Ashley and Robinson 1996), rivers with a breadth of at least 100 m (Angelone and Holderegger 2009) and urban area at least 100 m wide (Ray et al. 2002) as landscape barriers between adjacent localities. Golf fields were not included in the definition of biogeographic islands. The surface area was measured at 0.01-km^2 resolution and distance with a resolution of 1 m. Habitat was divided into four categories, namely "rice paddies", "fields", "forest" and "shrubs", each of which were identifiable from the maps at the selected resolution. The surface area for each habitat category was converted to a percentage of the surface area of the island. To be defined as a belonging to a specific type of habitat, each patch had to total a minimum of 500 m^2. Other landscape and climatic variables were not included in the analysis due to the narrow range of the selected area.

Each island was given a score of 1 for each 10 km^2 of surface area, a score of 1 for each habitat present, based on the main factors of importance (Wesche et al. 1987; Lomolino 1990), and a score of 1 if closer than 200 m from the next island, concurring with the range of yearly dispersion distance for hylids (Angelone and Holderegger 2009). The presence or absence of the focus species was encoded as 0 or 1, based on field surveys conducted between 15 May and 1 July 2014, matching with the breeding season of the species. Each site was surveyed once, following a transect line for a minimum of 15 min crossing the

expected adequate breeding area of the species, which has been determined as an adequate method to detect hylids (Sung et al. 2011). Descriptive statistics and habitat ranking were computed for each site, based on the scores attributed. We then used a logistic regression to measure the relationship between the occurrence of *Dryophytes suweonensis,* set as the dependant factor, and the five habitat variables, with the distance between islands and surface area set as independent continuous variables. The analysis was not run for *D. japonicus* due to the consistent occurrence of the species. The output of the analysis allowed using the probability scores as the predicted values of the dependent variable, hereby the occurrence of *D. suweonensis*. Subsequently, we tested the directionality of the relationship between the occurrences of *D. suweonensis* and the habitat types "rice paddy" and "forest" with a Spearman's rank-order correlation test. All statistical analyses were conducted in SPSS (ver. 21.0; SPSS Inc., Chicago, IL, USA).

RESULTS

The analysis of the biogeographic island within metropolitan Seoul accounted for a total of 19 sites (Fig. A3.1). The surface area ranged from 1 to 88 km² with a median value of 5.97 km². The distance between two islands ranged from 81 to 3,590 m, with a mean value of 685 m (SD =

902.90). The habitat type "forest" was represented by the highest frequency, being present for 17 out of 19 sites, while the two sites not displaying any forest were the only sites where rice paddies dominated. Fields and shrubs were accounted for at respective frequencies of 4 and 3 (Table A3.1). *Dryophytes japonicus* was present at all sites surveyed, and it was therefore not possible to discriminate the factor important for the occurrence of this species.

The habitat ranking ranged from 2 to 8, with high ranking habitats present at the lowest frequency, ranging from 6 to 2. More than half of the islands were characterised by a rank below or equal to 3 (Table A3.2). Unexpectedly, the presence of *D. suweonensis* coincided with the sites of lowest rank. The results of the logistic regression for the presence of *D. suweonensis* indicated that forest ($\chi^2 = 16.97$, df = 1, $p < 0.001$) and rice paddies (score=19.00, $df = 1$, $p < 0.001$) were statistically significant.

However, the distance between islands (score = 0.06, $df = 1$, $p = 0.784$), the surface area (score = 0.60, $df = 1$, $p = 0.440$), and the habitat variables "field" (score = 0.57, $df = 1$, $p = 0.452$) and "bush" (score = 0.37, $df = 1$, $p = 0.543$) were not statistically significant. The subsequent Spearman's rank-order correlation test showed that *D. suweonensis* was negatively correlated with "forest" ($r = -0.58$, $n = 19$, $p = 0.010$) but positively with "rice paddy" ($r = 0.99$, $n = 19$, $p < 0.001$).

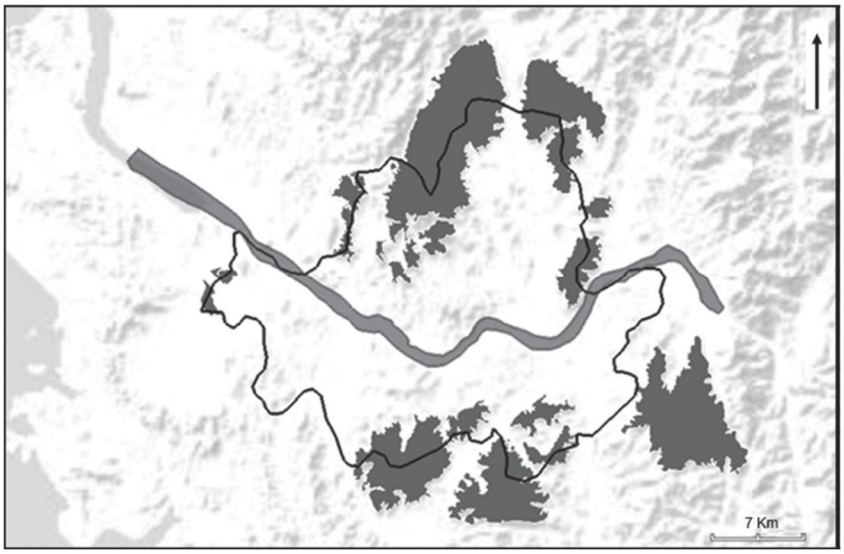

Figure A3.1. Spatial representation of the nineteen biogeographical islands of interest surrounding the city of Seoul. The dark black line is representative of the cadastral limits of the city, the central ribbon is the Han River and each of the grey patches is a biogeographical island. The name of the sites are in Table A3.1 and are set such as the first line of the table is the westernmost site, followed sequentially in clock-wise order.

Table A3.1. The 19 biogeographical islands within the cadastral city of Seoul. Dj stands for *Dryophytes japonicus* and Ds for *D. suweonensis,* encoded 0 for absence and 1 for presence. Dist. Stands for distance. The area is in square kilometres and the distance in metres. All landscape variables are given in percentage of ground cover.

Site	Surface area	Forest	Field	Shrub	Paddy	Dist.	Rank	Dj	Ds
Gonghang (S)	2.21	0	0	0	99.9	846	2	1	1
Gonghang (N)	7.64	0	0	0	100	846	2	1	1
Susaek-dong	3.33	97.15	2.85	0	0	81	4	1	0
Ganghyeun-dong	4.53	95.94	4.06	0	0	81	4	1	0
Bulkwang-dong	77.5	100	0	0	0	421	8	1	0
Hongeun-dong	0.78	100	0	0	0	421	2	1	0
Hongjae-dong	1.75	100	0	0	0	239	2	1	0
Muhak-dong	1.99	60.8	0	39.2	0	239	3	1	0
Samcheong-dong	4.55	78.9	0	21.1	0	146	4	1	0
Sanggye-dong	26.7	100	0	0	0	188	5	1	0
Cheonghak-ri	3.19	100	0	0	0	188	3	1	0
Junggye-dong	9.54	100	0	0	0	153	3	1	0
Mangu-dong	10.9	80.14	0.32	19.5	0	3,590	6	1	0
Geoyeo-dong	53.7	97.64	2.36	0	0	2,310	8	1	0
Taepyeong-dong	5.47	100	0	0	0	992	2	1	0
Gulyeong-san	5.97	98.32	1.68	0	0	277	3	1	0
Naegok-dong	6	100	0	0	0	277	2	1	0
Munweon-dong	31	100	0	0	0	260	5	1	0
Bisan-dong	35	100	0	0	0	1,460	5	1	0

DISCUSSION

Although the correlation between the surface of a protected area and the number of species was demonstrated for mammals (Newmark, 1987) and in general settings (Quammen 1996), it does not apply to hylids with narrower home ranges. Urban areas are typically characterized by a few biogeographic islands with low to high quality habitats, surrounded by urban environment. Seoul being a massively populated city, our results are consistent with the low biodiversity expected from a metropolis. Yet, two sites displayed high rankings, denoting a potential for the conservation of biodiversity within metropolitan Seoul.

These sites are adequate for *Dryophytes japonicus*, which appears to be a highly vagrant species for which the size, distance to the next biogeographic island and type of habitat are not critical for its occurrence. This translates into low requirements for breeding sites, and the appropriateness of any wetland for egg deposition and larval development. Oppositely, the presence of *D. suweonensis* at the two sites with the lowest ranking highlights the fact that biogeographic islands may benefit from diverse vegetation types, but specific features are required for some species. *Dryophytes suweonensis* does necessitate a specific type of habitat and seems not to be affected by the variety of

vegetation types, as long as rice paddies are available at the site.

Table A3.2. Ranking statistics for the analysis of optimal ecological conditions at nineteen sites within the city of Seoul. Sites are pooled by rank, with the highest ranks representative of habitats of higher quality.

Ranking	Frequency	% of sites per rank	Cumulative % age
2	6	31.6	31.6
3	5	26.3	57.9
4	2	10.5	68.4
5	3	15.8	84.2
6	1	5.3	89.5
8	2	10.6	100
Total	19	100	

Thus, biogeographic islands of all types should be conserved. Furthermore, this finding shows that a single habitat is required for *D. suweonensis*.

Increasing the habitat suitability of the biogeographic islands, with no *D. suweonensis* at present, would allow for the possible extension of the current *D. suweonensis* population. An increase in habitat suitability would involve the creation of artificial wetlands at the lowest elevation of other biogeographic islands on the green belt surrounding Seoul. Wetlands as small as 4 hectares are known to be suitable as potential hosts to *D. suweonensis* (Borzée and Jang 2015). These newly created wetlands, kept natural or artificial in the form of rice paddies, could therefore be an important addition to the value of the city of Seoul, and create a link between *D. suweonensis* populations north and south of the city, as the city is located at the heart of the geographic range of *D. suweonensis*. An increase in the habitat suitability would also mean the stabilisation of populations as the immigration rate decreases for habitats with a high suitability index, partially correlated to habitat ranking, due to saturation with species (Simon 2008). Equally, it would result in a higher colonisation rate and a potential range extension for the species, as biogeographic islands with high habitat ranking indices have a high emigration rate, correlated to the high number of dispersing individuals (Simon 2008). This positive effect would outweigh the current situation

for *D. suweonensis*, where all populations are located in biogeographic islands with low habitat rankings and are consequently subjected to higher probability of extinction due to ecological decay and chance events.

The beneficial aspect of population connectivity through wildlife corridors (Bennett, 1998) is shown by the landing strips of the international airport of Gimpo, between the two islands where *D. suweonensis* is present, acting both as an ecological buffer and corridor. Ecological corridors between the existing biogeographic islands with *D. suweonensis* and other sites where the species occurs would decrease the risk of genetic bottleneck, and lower the risk of extinction (Frankham 2005). Specifically, structures such as the eco-bridge in Namhyeon-dong (37.475582° N, 126.970795° E), which connects the geographic island of Bisan-3-dong northwards to a forested patch, would be adequate if the species occurs in one of these patches (Semlitsch 2008). The creation of ecological corridors and the resulting increased connectivity would enhance the movement of species between patches of natural habitats (e.g., Jordán et al. 2003). Accordingly, Kong et al. (2010) made a list of habitats used for connectivity in urban settings. The patches of importance, classified by impedance factor (*sensu* Opdam 1991) were scenery forests, public parks, riparian green space lining water bodies, green buffer corridors (e.g. protecting high-voltage transmission lines),

nursery areas (supplying saplings for urban greening), plazas, roadsides, agricultural sites, lands used for transportation and finally, open water. From these features, the riparian green spaces are of main importance to the city of Seoul. The city is located on flood plains that were seemingly used to be the adequate habitat for *D. suweonensis* before urbanisation (Won 1981). The creation of a row of vegetation matching the needs of *D. suweonensis* along these streams would greatly improve connectivity, in addition to the possibility to provide secondary advantages such as enabling carbon capture and improving the aesthetic aspects of the city. We suggest a hedge composed of a row of Korean willows (*Salix koreensis*) on the ground, paralleling a line of high grasses such as *Elymus repens* or *Phragmites communis*, further enlarged by other bushes, due to the known positive interaction between *D. suweonensis* and these species (Borzée and Jang 2015).

However, the development of corridors from and towards endangered species should be carefully planned due to the possible transmission of pathogens (Tabor et al. 2001), especially in the light of the presence of *Batrachochytrium dendrobatidis* in the area (Bataille et al. 2013). Ecological corridors should also be set in a way that does not increase the displacement of invasive species, such as the bullfrog *Lithobates catesbeiana,* which actively preys on smaller amphibians (Wu et al. 2005; Silva et al. 2009) such as *D. suweonensis*. Although

stochastic events such as geological changes have to be considered, the highest threat is present in the short term in the form of the urban development on the edge of the metropolitan city of Seoul. The protection of these sites by internationally recognised institutions such as the RAMSAR convention would provide benefits for both the conservation of the species (Kleijn et al. 2014), and possible sources of income through eco-tourism (Eagles et al. 2002).

PART II: BEHAVIOURAL ECOLOGY

This part contains all aspects of behavioural ecology in relation with the endangered status of amphibians. This includes variations in behaviour between species (chapter 4), niche segregation between species (chapter 5), competition between species (chapter 6 & appendix 4), relationship between agro-environmental variables and breeding behaviour (chapter 7), brumation and hibernation behaviours, in relation with migration and microhabitat use (chapter 8 & appendix 6), and mating behaviour (appendix 5).

Chapter four

VARIATIONS IN BOLDNESS, BEHAVIOURAL AND PHYSIOLOGICAL TRAITS OF AN ENDANGERED AND A COMMON HYLID SPECIES FROM KOREA.

ABSTRACT

Animal populations with narrow distribution ranges due to recent constrictions are predicted to be physiologically and evolutionarily less fit than their counterparts with wider distributions. In anurans, muscle mass and body size are generally correlated with locomotor and physiological performances. These, in turn, may be representatives of species specific strategies such as predator avoidance, exploration, habitat use, and dispersal. We conducted behavioural experiments and field observations to assess the differences in physiological capacity, exploratory behaviour and response delay between the endangered *Dryophytes suweonensis* and the widespread *D. japonicus*. The complementary genetic analyses revealed a lower genetic diversity in *D. suweonensis* than in *D. japonicus*. Despite its smaller size, *D. suweonensis* displayed a significantly larger mass for locomotor muscles, but a longer delay for responses to stimulus and a lower endurance than *D. japonicus*. Furthermore, *D. suweonensis* was on average, but non-significantly, less exploratory but faster than *D. japonicus*. The combined lower cognitive delay for behavioural responses

of *D. suweonensis* and the better endurance of *D. japonicus* make the later species more fit to its environment, allowing for faster escape and wider dispersal capabilities. Our results therefore highlight the correlation between the endangered *D. suweonensis* with a narrow ranges and depressed boldness, in comparison to the wide-spread and closely related *D. japonicus*. These results are clade specific but open a potential new line of research onto species decline.

INTRODUCTION

Small and isolated populations run higher risks of extinction than large populations (Shaffer 1981; Schoener and Spiller 1992; Ouborg 1993; Lynch et al. 1995; Jacquemyn et al. 2007). Accordingly, the risk of extinction is first related to demographic, genetic and environmental stochasticity (Gomulkiewicz and Holt 1995; Primack 2006). For instance, genetic erosion through genetic drift results in the loss of genetic polymorphism (Rodríguez-Zárate et al. 2013; Rivera□Ortíz et al. 2014) and in increased inbreeding (Jacquemyn et al. 2007; Barrett et al. 1991; Ellstrand and Elam 1993; Templeton and Read 2013).

Among others, population fitness is a function of effective population size and inbreeding depression (Keller and Waller 2002; Brommer et al. 2015), which directly impacts population persistence

(Reed and Frankham 2003; reviewed by Reed 2005). Bottlenecked populations often undergo high rates of fixed deleterious alleles (Whitlock 2000; Balick et al. 2015) and some of the consequences include biased mate selection (Houde 1988, 1994; Sato et al. 2014), decreased genetic and physical adaptation to the environments (Swallow et al. 2009), and depressed cognitive abilities (Fareed and Afzal 2014). For instance, the small wolf population on Island Royale (*Canis lupus*) suffers from congenital bone deformity due to inbreeding (Räikkönen et al. 2009), and inbreeding has a negative effect on growth and reproductive traits in rainbow trout (*Oncorhynchus mykiss*; Su et al. 1996).

Under restrictive environmental conditions, the naturally-occurring stochastic process of genetic drift leads to the rarefication of allelic variants, with bottlenecks acting as "haplotype selectors", and consequently selecting traits. This is the natural equivalent of laboratory line selections, where fitness traits may be positively or negatively selected. It is for instance the case of the inhibited immune system of "nude mice" (Fogh and Giovanella 2014), the 50 % decrease in hind limb muscle mass in mice (Hannon et al. 2008) and a running trait in mice (Swallow et al. 1998; Swallow et al. 2009). Selection can also lead to environment-specific fitness effects, such as the selection of predation enhanced behaviours in mice (*i.e.* lower boldness; Gammie et al. 2003).

Personality traits such as boldness and exploration (*sensu* Reale et

al. 2007) are related to growth, reproduction rate, range extension and survival (Sih et al. 2004a; Sih et al. 2004b; Bell 2007; Reale et al. 2007; Smith and Blumstein 2008; Cote et al. 2010; Maes et al. 2012). For instance, Brodin et al. (2013) demonstrated inter-population variations in personality traits for the common frog (*Rana temporaria)*. Both tadpoles and froglets from island populations were bolder and more exploratory than conspecifics from mainland populations. Besides, interspecific differences in personalities (Dall et al. 2004) can explain behavioural variations in relation to population dynamics at the species level. Furthermore, some of these species specific traits, such as calling length or response latency to stimulus are correlated with fitness (reviewed by Briffa and Weiss 2010). Finally, divergent evolution of species for fitness related traits, such as morphology, also relates to ecological diversification (Rezende and Diniz-Filho 2012). For instance, fossorial anuran species are adapted to subterranean life and thus have shorter limbs, and consequently lower jumping performances than that of arboreal species (Gomes et al. 2009). This provides a fitness advantage to one of the two species when facing the same threat.

The Korean peninsula is inhabited by two treefrogs species, *Dryophytes suweonensis* and *D. japonicus*, in sympatry on the whole range of the former. The two species belong to the *Dryophytes japonicus* group (sensu Hua et al. 2009, previously assigned to *Hyla;* Duellman et al.

2016), and diverged between 2 and 7.1 may (Yang et al. 1981; Lee and Park 1992 and Lee et al. 1999; Li et al. 2015; Dufresnes et al. 2016). *Dryophytes suweonensis* has been designated as endangered by the Ministry of Environment of the Republic of Korea (2012) and by the IUCN (2014). The original distribution of the endangered *D. suweonensis* has been decreasing over the last decades, while the original distribution of *D. japonicus* did not (BORZÉE et al., 2017). Besides, the population trend for *D. suweonensis* is negative, based on field surveys (Borzée et al. 2017d) and genetic analyses (Borzée et al. 2015). The two species are syntopic, inhabiting the same microhabitats although separated by temporal segregation in habitat use (Borzée et al. 2016a; Borzée et al. 2016b). Male *D. japonicus* are about 5.18 % longer in snout-to-vent length than male *D. suweonensis* (Borzée et al. 2013). The head of *D. japonicus* is on average broader than *D. suweonensis,* hypothetically correlated with variations in diet. The aim of this study was therefore to investigate differences in morphological, physiological and behavioural traits between the two Hylid species.

Due to the negative relation between morphological, physiological and cognitive traits in small populations, we argue that the drop in range and population sizes in *D. suweonensis* resulted in inbreeding depression, leading to a decrease in physiological abilities. This opposition with the expectation for *D. japonicus* is predicted to be visible through behavioural

traits. Specifically, here we hypothesise that the species on the limited geographic range, *D. suweonensis*, will express a less vigorous behaviour than the one on the large range, *D. japonicus*, due to differences in behaviour. Besides, we expect to see a negative correlation between vigour and haplotypes diversity. We tested our hypothesis and expectation through field observations and two sets of open arena experiments for the two species. For both observations and experiments, we characterized a suite of variables for morphology, physiological abilities and behavioural traits. Besides, we conducted a haplotype analysis for a single mtDNA gene, as a proxy for genetic diversity.

MATERIAL AND METHODS

Animal collection and maintenance

Both treefrog species were collected from six rice-paddy complexes in the city of Paju, Gyeonggi-do, Republic of Korea (Table 4.1). Adjacent complexes were separated by at least 2000 m or landscape barriers that are not permeable to treefrogs (Roh et al. 2014). This study was approved by the Ministry of Environment of the Republic of Korea (permit 2013-16), and was carried out in accordance with the approved guidelines. The research permits limited sampling for *D. suweonensis* to 10 % of the population at a site, and up to a total of 30 individuals for this study. Accordingly, the night before the behavioural experiments was

spent conducting surveys to assess the population size at focal sites (Borzée et al. 2017d). Species identification was assessed through advertisement calls (Jang et al. 2011; Park et al. 2013). Individuals used for the behavioural experiments were caught the day subsequent to the surveys, between 16 May and 7 June 2013.

Thirty *D. suweonensis* (SVL = 2.98 ± 0.19 cm, mean ± SD) and 60 *D. japonicus* (SVL = 3.18 ± 0.29 cm) were caught for this study. After experimentation, each individual was measured for SVL, and non-invasive DNA sampling was conducted with buccal swabs (Broquet et al. 2007) for species confirmation. Following ethical guidelines, individuals were kept for a maximum of 24 h, prior to release at the sampling location. In between experimental trials, frogs were kept in individual housings at room temperature, and at a minimum of 40 % relative humidity.

Genetic analysis

Mitochondrial DNA was extracted from freeze preserved swabs (-20°C) using an Enzynomix DNA Extraction kit, following the instructions of the manufacturer (Enzynomix; Daejeon, Korea). Mitochondrial markers for Cytochrome C oxidase subunit I (LepF1: 5'-ACC AAT CAT AAA GAT ATT GGT-3' and LepR1: 5'-CCT CTG GGT GTC CGA AAA ATC A-3'; Hebert et al., 2004) were selected to perform the molecular analyses, following the protocol developed by Jang et al. (2011). PCR

reactions contained 1 µl of DNA (adjusted to 20 ng DNA/µl) and amplifications were carried on a PTC-100 thermocycler (BIO-RAD; Applied Biosystems; Foster City, USA). PCR reactions were then sent for purification and sequencing using both forwards and reverse primers to Macrogen Inc. (Seoul, Korea) on an ABI PRISM 3100 automatic sequencer (Applied Biosystem Inc.; USA).

Sequences were aligned and trimmed using Geneious v. R9 (Biomatters Ltd; Auckland, New Zealand), and the data analysed through haplotype comparison and genetic distances. The number of haplotypes for each species was assessed through the creation of haplotype networks, computed through median joining network calculations in Network (v 5.0; Fluxus Technologies Ltd., Germany) with all parameters set to default. Genetic *p*-distances (pairwise deletion) and standard error were calculated using Mega v7 (Tamura et al. 2013; Kumar et al. 2016), prior to comparisons. The calculations were conducted with 1000 bootstraps, with the substitution model including transitions and transversions.

Experimental setup for behavioural trials

Two sets of behavioural experiments were conducted: "hold and release" and "drop and swim", with a constant set of environmental variables between the two experiments. An indoor experimental space was replicated at each of the experimental sites (Fig. 4.1). The indoor arena was an acrylic tank (100 x 30 x 40 cm), filled with chemical-free water up

to 10 cm at room temperature (23.46 ± 1.07 °C, mean ±SD) for the type if experiment requiring it.

Table 4.1. Localities, dates and housing abiotic variables for individuals caught for the behavioural experiments. All samples were caught at six independent sites in the area of Paju, Gyeonggi Region, Republic of Korea. Temperature is in °C, light intensity in lux, sound level in dB and humidity in percent.

Site	GPS coordinates		Light intensity (lux)	Temp air (℃)	Temp water (℃)	Sound level (dB)	% Hum.
	Lat.	Long.					
Papyeong	37.933	126.852	16.28	21.86	23.50	53.25	41.78
Gimpo	37.683	126.628	23.33	24.00	25.00	54.67	70.07
Munsan	37.853	126.773	98.07	22.58	22.50	52.67	58.74
W. Imjingack	37.885	126.746	123.00	23.60	22.50	54.00	60.55
E. Imjingack	37.902	126.770	120.50	23.00	22.75	53.25	58.65
Geumcheon	37.853	126.773	125.50	24.27	24.50	53.43	56.66

To record lateral and dorsal views during the experiments, and to ensure that velocity was measured independently of the direction of movements, a mirror was used. It was set leaning at 45˚, with the centre of the two diagonals 30 cm above the centre of the arena.

Ambient conditions were controlled for temperature, humidity (HT-350 thermo hygrometer; Iondo; Seoul, Republic of Korea), luminosity (Lux/Fc Light meter TM-201; Tenmars; Taipei, Taiwan) and sound (sound level meter, Cat. No 33-2055; RadioShack; Texas, USA) in the arena (Table 4.1). All experimental trials were recorded using a video camera (Sony DCR-TRV30 NTSC, Tokyo, Japan). To reduce perspective and parallax errors, the camera was located three meters away, the lens opening parallel to the arena, with a reference available for distance and body measurements. The order in which individuals were assigned to the two experiments was randomised.

"Hold and release" experiment

The "hold and release" experiment was designed in order to measure the temporal and behavioural responses to a novel stimulus. Each individual was placed under an obscured hide at the centre of the arena and left still for 1 min, under the experimental conditions (Table 4.1; Fig. 4.2). The hide was then lifted vertically by pulling a string through a knob and removed from view, in a constant fashion from trial to trial. The experimental trial ran for a maximum of ten min. For individuals climbing

out of the arena before the end of the experiment the last recorded frame was the one when the frog reached the open top, before escaping. An individual was used only once in each experimental trial. If a frog was found holding on the hide (11 out of 90 cases), or if it was partially displaced immediately following the lift of the hide (6 out of 90 cases), the individual was removed from the trial before being reinterred for random selection after a minimum 2 h.

From this experiment we measured the time elapsed until the first body movement and displacement, defined as the distance moved during the trial. To measure the distance moved, we used a reference point on frogs' dorsal side, such as a clear pattern unmistakably recognizable. We also measured the number of steps, the number of bouts of activity, the number of steps per bouts, the number of jumps and the number of jumps per bouts. Finally, we measured the time elapsed to reach the edge of the arena, the total time spent in movement, and the distance travelled.

"Drop and swim" experiment

This experiment was designed in order to measure the morphology when in activity and the physiological abilities of the two species. Frogs were individually released vertically, 34.64 ± 3.13 cm above water in the arena (Fig. 4.2).

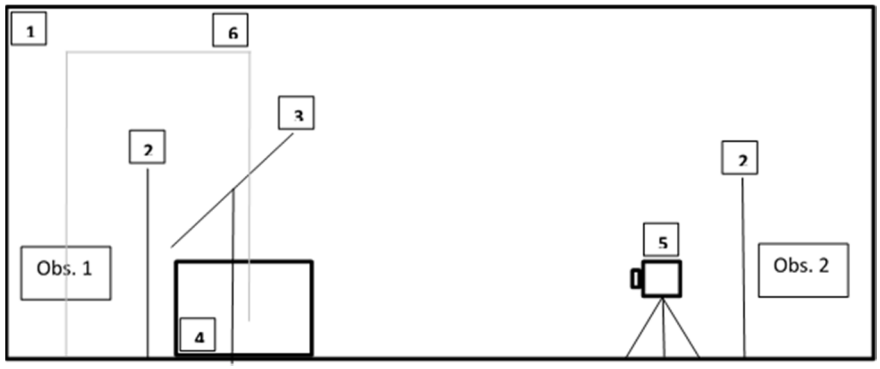

Figure 4.1. General experimental setting for both of the experiments. The external frame is the 3D external isolation (depth not represented). Both "Obs." represent the position of the experimenters. 1: 3D external isolation out of white fabric surrounding the open arena to prevent external influences. 2: white panes behind which the experimenters hide, 3: mirror allowing for the study of displacement in 3D, 4: open arena, 5: video recorder, 6: knob allowing for displacement of objects within the open arena without human intervention to minimise interactions.

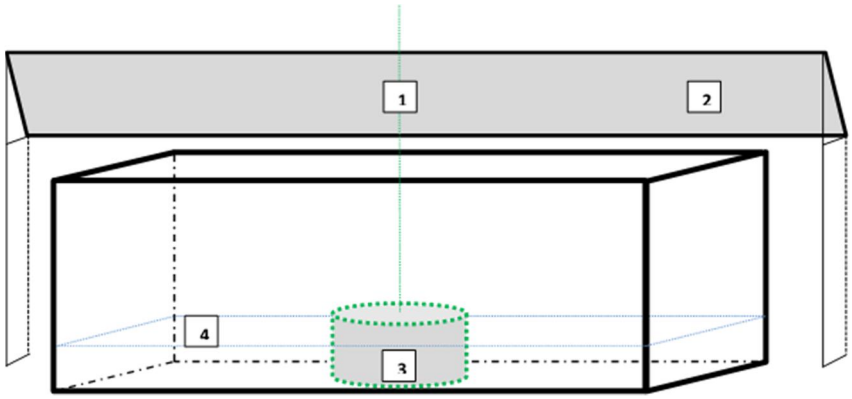

Figure 4.2. Experimental setting seen from the lens of the camera (see Fig. 4.1), the hide (green) was used for the "hold and release" experiment and the tank (100x30x40 cm) filled with 10 cm of water for the "drop and swim" experiment (blue). 1: knob allowing for displacement of objects within the open arena without human intervention to minimise interactions. 2: mirror for vertical view. 3: position where the individual is introduced in the "hold and release" experiment. 4: position where the individual is introduced in the "drop and swim" experiment.

The movements of each frog were recorded from the moment the frog was released, until 10 s after the individual reached any of the vertical sides of the tank. After each trial, the individual was removed from the arena, the water was changed and the tank wiped with 100 % ethanol. For this experiment we measured the maximal leg elongation and width, the response time, the total number of swimming strokes to reach the edge of the arena, the distance covered by each stroke and the time elapsed per stroke, in order to calculate velocity per stroke, and averaged, for each individual. Due to improvements in experimental design, the first ten individuals for which data were recorded had to be removed from the data set.

Field calling activity

To confirm the natural occurrence of the results obtained from the behavioural experiments, field observations were conducted on the northern range of *D. suweonensis* distribution (Borzée et al. 2016c). Site selection was driven by the permits agreed upon by the Ministry of environments (2015-03, 2015-05, 2015-6 and 2015- 28), resulting in 17 independent sites, although recordings were acquired at 14 sites (n = 54) for *D. suweonensis* and 12 sites (n = 53) for *D. japonicus* (Table 4.2). Upon arrival at the site, cameras (ES95; Samsung; Seoul, Republic of Korea) were quietly deployed in front of randomly chosen individuals, for which the species was identified through calls (Jang et al. 2011; Park et al.

2013). Each camera was set 40 cm high on a wooden stick, placed 35 to 40 cm in front of the calling individual, and fitted with an LED (Point LED light model # 4984343805309; Hiroshima, Japan). The cameras were set to record for 15 min upon positioning.

Recordings started as soon as the cameras were set and stable, from which point the data extraction started. Some frogs did not call during the 15-min recording period and hence were not included in the analysis. We tested for difference in the number of non-calling individuals between species (*D. japonicus* $n = 54$; *D. suweonensis* $n = 46$) to prevent any sampling bias, and no significant difference was detected (Likelihood ratio test; $\chi = 0.82$, $df = 1$, $P = 0.365$). From the video, we collected the occurrence of movements, the time elapsed until the first call production ("silence pre-call", in seconds), the average length of calling bouts ("calling period", in seconds) and the average length of silences between calling bouts ("silence period", in seconds).

A call bout was defined as a continuous calling period uninterrupted by more than six seconds, as matching with a retraction of the calling sac, and approximately matching with the definition of call bouts used by Tobias (2011). Additionally, we collected day or night time during the observations ("photoperiod") based on the detection of the light halo created by the LED attached to the camera support (binary encoded for the analysis).

Table 4.2. Localities where field work was conducted for the observations. The maximum of recording per site is 18 for *Dryophytes suweonensis* (14 sites) and 13 for *D. japonicus* (12 sites).

Site	Latitude	Longitude	*D. japonicus* recordings	*D. suweonensis* recordings
1	37.0402	126.8752	2	0
2	37.8985	126.7586	1	1
3	36.8080	126.9936	0	5
4	36.9460	126.9337	7	1
5	36.9447	126.8957	0	5
6	36.2474	126.8490	0	18
7	37.7956	126.8014	13	8
8	35.9914	126.9474	0	4
9	37.6257	126.6720	9	1
10	37.8105	126.7889	6	2
11	37.8179	126.7077	3	1
12	37.8868	126.7436	3	0
13	37.7641	126.6939	2	0
14	36.8856	126.9267	0	2
15	36.8186	126.8812	3	1
16	35.9897	126.9298	3	2
17	36.9895	127.7739	1	3

Data extraction

For both sets of behavioural experiments, data were extracted from the recordings under blind condition: each data set was extracted without information regarding the species of the individual on the video. Videos were analysed with Tracker (v4.62; Open Source Physics, Free Software Foundation; Boston, USA) for the event sequences and time elapsed while all distances were extracted with ImageJ (v1.48; National Institutes of Health; Bethesda, USA).

The following data were extracted from the "hold and release" experiment, with T_0 defined as the moment when the hide was removed, and T_f as the terminal of a trial: "first movement t": latency to the first movement after T_0 (including body movements without motion from the point of origin but not including motion from the point of origin), "first displacement": latency to the first motion from the point of origin, "Total number of head movement": total number of head movement without body motion from T_0 to T_f, "Average number of steps per bouts": average number of step per bout (averaged over several bouts for an individual to prevent pseudo replication), "Average number of jumps per bouts": average number of jumps per bout (averaged over several bouts for an individual to prevent pseudo replication), "Total time travelled": time spent in movement, "Distance travelled": total distance covered by a frog before T_f, "Time to reach the tank's edge": time from T_0 to the moment

the frog reached the edge of the tank.

From the "drop and swim" experiment, the following measurements were extracted: "maximal leg extension": maximal leg extension, "maximum leg width": maximum leg width of the upper leg muscle while in extension, "response time": time in second between when the frog reached the water and when it initiated the first stroke, "total number of strokes": total number of strokes between the dropping point and the moment the frog stopped moving, "averaged velocity per stroke": average velocity of strokes for an individual, calculated as the average distance per stroke divided by the average duration of a stroke and "velocity first stroke": velocity of the first stroke, being critical to escape predators. All values were averaged by individual to prevent pseudo-replication, as this controlled for the very unbalanced number of events recorded from different individuals.

Statistics

The results of the two behavioural experiments were analysed separately because data extraction from the "hold and release" experiment was based on 30 *D. suweonensis* and 60 *D. japonicus*, while the dataset on "drop and swim" experiment was based on 30 *D. suweonensis* and 50 *D. japonicus* (Table 4.3). Due to the high number of correlated variables: 17 out of 28 variables for the "hold and release" experiment ($R^2 \leq 0.80$, $p \leq 0.047$) and 7 out of 15 variables for the "drop and swim" experiment ($R^2 \leq$

0.72, $p \leq 0.08$); both data sets were submitted to Principal Component Analyses with the species as discriminant factor and all other variables as dependent variables. An eigenvalue of 1 was set as the threshold of significance for the principal components (PCs), which were extracted based on the component score (see Table 4.4 and 4.5). The resulting PCs for each dataset were then submitted to one-way ANOVAs to assess their independence by species. Because of unclear video signals during data extraction, a total of 19 datapoints for the "hold and release" dataset and 41 datapoints for the "drop and swim" dataset could not be extracted, out of a total of 2520 and 1200 datapoints respectively. ANOVAs on the PCs were selected as fulfilling all assumptions. Namely, the observations were independent, there were no outliers when verified through the analysis of boxplots, and the data was normality distributed when tested through a Kolmogorov-Smirnov test of normality: $D > 0.12$, $df = 58$, $p > 0.005$ for the "drop and swim" dataset, and $D > 0.12$, $df = 72$, $p > 0.005$ "hold and release" dataset.

Each dataset was subsequently analysed separately through a Binary Logistic Regression for a reduced number of variables. The variables were selected due to their importance as loading factors in the previous PCA, and only if they were not correlated with other variables to avoid multicollinearity. For both datasets, the dependent variable was species. For the "hold and release" dataset the covariates were first

movement, total time travelled, and time to reach the tank's edge. Besides, these variables were adequate summary variables for boldness and exploration. For the “drop and swim" dataset the covariates were maximum leg width, response time, and average velocity per stroke. When testing for the assumptions, there was one or two outliers per variables for both datasets, but as these outliers were close to the whiskers’ extremities in the corresponding boxplots, and as values were biologically relevant, their presence was not estimated to be a significant bias. Besides, for each of the dataset separately, the selected variables were not collinear as displaying tolerance values > 0.1 and Variance Inflation Factor values < 10. Finally, for each of the dataset separately, there was a linear relationship between the continuous independent variables and the logit transformation of the dependent variable, tested through the Box and Tidwell (1962) procedure with Bonferroni corrections (Tabachnick and Fidell 2014), with $p > 0.005$ for all variables and thus rejecting the null-hypothesis.

The analysis of the observation data was ran through a Binary Logistic Regression, due to the type of variables selected for the model. Species was the dependent variable, and moving, photoperiod, calling period, silence pre-call and silence period were covariates, with moving and photoperiod set as categorical variables. Silence pre-call was biologically significant here as related to boldness. However, the variable

was correlated to moving (Pearson correlation test; $r = 0.26$, $n = 91$, $p = 0.015$) and to calling period (Pearson correlation test; $r = 0.28$, $n = 56$, $p = 0.034$). Thus, to be able to integrate this variable in the model nonetheless, we ran the regression with 1000 bootstraps and a confidence level of 95 %. No outliers were detected for any of the variables, and there was a linear relationship between the continuous independent variables and the logit transformation of the dependent variable, tested such as above. All analyses were performed with SPSS 21.0 (SPSS, Inc.; Chicago, USA).

RESULTS

Genetic analysis

Amplification was successfully conducted for 47 *Dryophytes japonicus* and 22 *D. suweonensis*, for a total of 435 bp, confirming the species identification (GenBank submission BankIT ID 2067894 and 2067916, respectively). The haplotype network analysis led to 20 haplotypes organized in star networks around two central haplotypes in *D. japonicus*, and 7 haplotypes organized in a generally linear fashion in *D. suweonensis*. Thus, once controlled for the number of individual, the genetic diversity ratio was higher for *D. japonicus* (0.42) than for *D. suweonensis* (0.31), representative of a very likely genetic bottleneck for *D. suweonensis*. The genetic *p*-distances were different within the two species with a genetic diversity four folds larger in *D. japonicus* (0.009 ±

0.003) than for *D. suweonensis* (0.002 ± 0.001). For comparison and scale, the within group mean distance was 0.128 for *D. suweonensis* and 0.015 for *D. japonicus*, while the overall mean distance was 0.061 ± 0.007.

"Hold and release" experiments

The first experiment was designed to measure the temporal response to a novel stimulus. The largest difference between the two species was for the time to reach the tank's edge, for which *D. japonicus* was 101.69 s faster. Similarly, *D. suweonensis* took 22.89 s longer than did *D. japonicus* to initiate the first movement. However, the difference between the average times to the first displacement between the two species was 17.56 s, *D. japonicus* being faster than *D. suweonensis* for this variable as well (Fig. 4.3).

The PCA on the "hold and release" experiment provided two PCs (Table 4.4). Among the loading factors > 0.5, the five principal variables loading in PC1 were: total number of head movement, average number of steps per bouts, average number of jumps per bouts, total time travelled and distance travelled.

Table 4.3. Descriptive statistics for the two treefrog species, *Dryophytes japonicus* and *D. suweonensis*, limited to the variables loading in the PC and therefore used for the analysis. The table contains data for both "hold and release" and "drop and swim" experiments.

	Dryophytes japonicus		*Dryophytes suweonensis*	
	Mean	SD	Mean	SD.
"hold and release" experiment				
1st movement	29.43	45.03	52.32	56.82
1st displacement	80	69.71	97.56	80.2
Total number of head movement	0.94	1.32	1.12	1.53
Average number of steps per bouts	5.57	5.5	3.76	2.12
Average number of jumps per bouts	1.4	2.09	0.68	1.11
Total time travelled	8.53	9.41	9.42	6
Distance travelled (cm)	61.2	48.9	55.84	39.75
Time spent to reach the tank's edge	154.47	97.65	256.16	135.8
"Drop and swim" experiment				
Maximal leg extension (cm)	4.64	0.99	4.81	0.72
Maximum leg width (mm)	0.5	0.06	0.56	0.06
Response time	1.31	1.85	0.99	1.67
Total number of strokes	4.97	2.37	4.85	2.46
Average velocity per stroke (m/s)	73.29	60.21	77.06	40.42
Velocity of the first stroke (m/s)	11.68	6.83	16.63	8.46

Time is in sec.

For PC2, the principal loading variables were: first movement, first displacement, Total time travelled and time to reach the tank's edge. The cumulated percentage of the two PCs accounted for 76.80 % of the total variation. PC1 was representative of exploration, because the factors were related to exploration such as the distance travelled, total time spend in movement and displacement speed. PC2 was representative of response time, because loaded with factors such as the time elapsed until the movement and displacement, and the time taken to escape the arena. The results of the ANOVA showed that PC2 was significantly different between the two species (Table 4.4), indicating a faster response to a novel stimulus for *D. japonicus*, in comparison to *D. suweonensis* (Table 4.3).

The Binomial Logistic regression to determine variations between the species was statistically significant, $\chi^2_3 = 12.74$, $p = 0.005$, although the model explained only 21.70 % (Nagelkerke pseudo-R^2) of the variance but correctly classified 68.0 % of cases. The only significant difference between the two species was for time to reach the tank's edge (Wald = 7.74, $df = 1$, $p = 0.005$), as first movement (Wald = 0.17, $df = 1$, $p = 0.681$) and total length travelled (Wald = 0.05, $df = 1$, $p = 0.825$) did not reach significance.

"Drop and swim" experiment

The PCA on the "drop and swim" experiment resulted in three

PCs. The PCs were loaded by 1 to 3 factors, accounting for 78.70 % of the variation (Table 4.5).

Table 4.4. PCA on the dataset from the "hold and release" experiment. An eigenvalue of 1 was set as the threshold of significance for the PCs. Loading factors were reported as important if > 0.5. The two resulting PCs were then submitted to a one-way ANOVA to assess their independence by species.

Principal components	1- Exploration	2- Response time
First movement	-0.232	0.723
First displacement	-0.391	0.752
Total number of head movement	0.510	0.324
Average number of steps per bouts	0.746	-0.010
Average number of jumps per bouts	0.569	0.060
Total time travelled	0.749	0.421
Distance travelled	0.925	0.076
Time spent to reach the tank's edge	-0.142	0.825
Average number of steps per bouts		
Average number of jumps per bouts	2.78	2.06
Total time travelled	34.80	25.76
Distance travelled	34.80	60.55
ANOVA		
df	1,70	1,70
Mean Square	1.32	6.08
F	1.32	6.56
P-value	0.253	0.013

PC1 was linked to velocity, as the loading factors, maximal leg extension and velocity of the first stroke, engaged in speed production. PC2 was linked to physiological performances due to the loading factors: response time, total number of strokes and average velocity per stroke, factors linked to endurance; in opposition with speed busts factors from PC1. PC3 was linked to morphology because only loaded with hind leg maximal width.

The ANOVA on the three PCs provided significant difference for PC1 and PC3 (Table 4.5), and thus related to velocity and morphology, in correlation with muscle mass. If isolated from the PCs, the maximum leg width, a proxy to muscle mass (Rome et al. 1992), was significantly different between the two species (ANOVA; $\chi^2 = 0.06$; $F = 16.59$, $P < 0.001$). The maximum leg width was 0.56 ± 0.06 mm for *D. suweonensis* and 0.50 ± 0.06 mm for *D. japonicus*.

The Binomial Logistic regression to determine variations between the species was statistically significant, $\chi^2_3 = 17.32$, $p = 0.015$, and the model explained 35.70 % (Nagelkerke pseudo-R^2) of the variance, while correctly classifying 65.5 % of cases. The only significant difference between the two species was for maximum leg width (Wald = 9.84, $df = 1$, $p = 0.002$), as response time (Wald = 1.35, $df = 1$, $p = 0.244$) and average velocity per stroke (Wald = 0.01, $df = 1$, $p = 0.905$) did not reach significance.

Field calling activity

The results of the Logistic Regression to assess the difference in behaviour between the two species was statistically significant, χ^2_5 = 24.03, $p < 0.001$, and the model explained 47.20 % (Nagelkerke pseudo-R^2) of the variance, while correctly classifying 74.5 % of cases. The results showed that moving, photoperiod and calling period were significantly different between the two species (Table 4.6).

The number of individuals moving was higher for *D. japonicus* (37.3 %, n = 19) than for *D. suweonensis* (14.0 %, n = 6). The calling period was more than twice longer for *D. japonicus* (52.02 ± 66.17 s) than for *D. suweonensis* (25.67 ± 24.70 s). Finally, the 54 *D. japonicus* included in the analysis called at night, however, out of the 46 *D. suweonensis* recorded 13 called during day time.

DISCUSSION

Our results for both behavioural experiments and field observations were consistent with our predictions of a less vigorous behaviour in *Dryophytes suweonensis*, but a bolder behaviour in *D. japonicus*. Specifically, *D. japonicus* responded faster to novel stimuli and was more exploratory than *D. suweonensis*.

Table 4.5. PCA on the dataset from the "drop and swim" experiment. An eigenvalue of 1 was set as the threshold of significance for the PCs. Loading factors were reported as important if > 0.7. The three resulting PCs were then submitted to a one-way ANOVA to assess their independence by species.

Component Score Coefficient Matrix			
Principal components	1- Velocity	2- Physiological performances	3- Morphology
Maximal leg extension	0.741	-0.24	0.241
Maximum leg width	0.376	-0.196	0.802
Response time	0.061	0.748	0.367
Total number of strokes	0.593	0.707	-0.161
Average velocity per stroke	0.579	-0.612	-0.179
Velocity of the first stroke	0.879	0.16	-0.345
Initial Eigenvalues			
Total	2.15	1.55	1.01
% of Variance	35.88	25.92	16.86
Cumulative %age	35.88	61.8	78.7
ANOVA			
df	1,56	1,56	1,56
Mean Square	2.28	3.9	4.9
F	2.33	4.12	5.27
P-value	0.132	0.047	0.026

Table 4.6. Results of the Binary Logistic Regression to assess the variations in boldness related to the calling behaviour of the two species. Only the variables moving, photoperiod and calling period were significantly different between the two species.

	B	Bias	SD	*P*-value
Moving	1.43	1.69	8.74	0.029
Photoperiod	21.30	-0.73	3.98	0.005
Calling period	0.02	-0.02	0.10	0.047
Silence period	0.05	-0.03	0.36	0.500
Silence pre-call	0.01	0.00	0.01	0.730

The relationship between response time and boldness is exemplified by Mamuneas et al. (2014), where bolder three-spined sticklebacks (*Gasterosteus aculeatus*) make decisions faster than their shyer conspecifics. The difference in response time to the same stimulus between *D. suweonensis* and *D. japonicus* may indicate opposite extremes on the shy-bold continuum (*sensu* Sih et al. 2004a; Sih et al. 2004b; Reale et al. 2007). This in turn may relate to exposition to a wider variety of environments for *D. japonicus* than for *D. suweonensis*, as the first species migrates between two different environments to breed and hibernate, while the second species is confined to a single habitat, rice paddies, for both breeding and hibernation (Borzée et al. *in review*; Borzée and Jang 2016).

Our results also demonstrate that the extended thigh of *D. suweonensis* is significantly wider than that of *D. japonicus*. As the physiological capacities of anurans directly correlate with muscular mass (Hirano and Rome 1984), it is fitting that *D. suweonensis* displays a higher average velocity per stroke than *D. japonicus*. Anurans recruit all of their extensor muscle function during locomotion and *D. suweonensis* is therefore faster while swimming, as the distance a frog travels is directly proportional to power production (Hirano and Rome 1984). These results are unanticipated as the snout-vent length of *D. suweonensis* is on average 5.18 % smaller than that of *D. japonicus* (see Borzée et al. 2013

for details), and thus *D. japonicus* was expected to have a larger body mass. It was hypothesized that the population decline in *D. suweonensis* would be a result of the inferior competitive ability due to a smaller size (Wassersug and Sperry 1977; Emerson 1978; Walton 1988), however, this hypothesis was only partially supported and may not apply to all types of habitats. Because *D. suweonensis* breeds at the centre of water bodies (Borzée et al. 2016a; Borzée et al. 2016b), factors such as breeding ecology may have led to the development of muscles related to swimming abilities. By contrast, *D. japonicus* produces mating calls from the bank of rice paddies and is consequently not required to swim, at the exception of females during amplexus. Variations in swimming capacities may therefore be the by-product of differential microhabitat use in calling between the two species (Borzée and Jang 2015). However, the fact that *D. suweonensis* was found calling during daytime suggests a mal-adaptive behaviour, potentially resulting from ecological character displacements. This could be resulting from a competitive disadvantage compared to *D. japonicus*, with the bold species activities resulting in a modification of the shyer species' behaviour.

The variation in swimming capacities may also be the result of different predation pressure. *Dryophytes japonicus* is present on the edge of rice paddies (Borzée et al. 2016a), and thus more often exposed to predation by Colubridae (Webb et al. 1962) and other snake species

competing for preys in rice paddies (Mori 1988; Kadowaki 1992). On the other hand, as *D. suweonensis* is present inside rice paddies, the species may more often fall prey to wading birds such as herons and egrets (Fujioka and Lane 1997; Hirai and Matsui 2000). Besides, variations in response time can originate from escape behaviours: predation by snakes is best avoided though mimicry and immobility (exemplified by Cooper et al. 2008 for *Craugastor* frogs and Martín et al. 2005 for *Rana perezi*), and is the common anti-predation behaviour used by hylids (Toledo et al. 2010). Oppositely, wading birds typically scan and seek for prey, and are better escaped by escaping and hiding under water plants (Bisazza et al. 1998).

Finally, because the decrease in population size leads to inbreeding depression (Keller and Waller 2002; Brommer et al. 2015), we expected a less vigorous behaviour for *D. suweonensis*. Star networks denote recent population expansions, and higher numbers of haplotypes are representative of genetic variability. Besides, the larger mean distance within group for genetic diversity in *D. suweonensis* is potentially explained by a reduced gene flow. Thus, the comparatively bolder behaviour of *D. japonicus* matches with the results of the genetic analyses. A lower fitness for small population has been illustrated in cheetah through the weakening of the immune system activity (O'brien et al. 1985), and can be compared to the difference sensitivity to pollutants

by the two *Dryophytes* species (Borzée et al. in press).

This assessment of a bolder personality for the species with the larger geographical range is matching with previous studies on several species, including amphibians (Dall et al. 2004; Maes et al. 2012; Brodin et al. 2013). The difference in distribution ranges between the two hylid species may be a consequence of the variation in boldness, allowing *D. japonicus* to colonize and settle on a broader range than *D. suweonensis* (see Kuntsi et al. 2006). This hypothesis is supported by Pittman et al. (2014), in which individuals with higher boldness are better at long distance dispersal. This is due to a lower responsiveness to good quality habitat, as dispersing individuals do not settle in the first adequate habitat encountered. Good quality habitats will attract most individuals, but only the boldest will keep on looking for an even better one.

Chapter five

TEMPORAL AND SPATIAL DIFFERENTIATION IN MICROHABITAT USE: IMPLICATIONS FOR REPRODUCTIVE ISOLATION AND ECOLOGICAL NICHE SPECIFICATION.

ABSTRACT

Niche differentiation enables ecologically similar species to coexist by lessening competition over food and/or shelters and may be critical for reproductive isolation between closely related species in close proximity. Because no extra traits need to evolve, spatial and temporal differentiation may readily take place to complement other isolating mechanisms. Two closely related treefrog species occur together in Korea: the endangered *Dryophytes suweonensis* and the widespread *D. japonicus*. Advertisement calls are differentiated, but it is unclear whether call difference is sufficient for reproductive isolation. We tracked individuals of both species to study fine-scale differentiation in microhabitat use in the diel cycle of the breeding season using a Harmonic Direction Finder. Tracking male movement patterns of both species revealed spatial and temporal differentiation in microhabitat use for calling and resting during the breeding season. Males of both *D. suweonensis* and *D. japonicus* occurred in all five microhabitats identified in this study: rice paddy, ground, buried, grass, and bush. Both treefrog species showed general

similarities in calling from rice paddies and resting in grass and bush. However *D. suweonensis* moved into rice paddies and produced advertisement calls three hours earlier than *D. japonicus*. These differences likely minimize contact between the species and provide an additional isolating mechanism. Additionally, the activity of *D. suweonensis* may be contributing to the decline of this species, as resting in grass would increase dangers from predatory birds and habitat disturbance.

INTRODUCTION

Prezygotic reproductive isolation between two closely-related taxa is generally favoured over postzygotic isolation, due to prevention of gamete waste (Fouquette 1960; Lemmon 2009). There are several mechanisms of prezygotic reproductive isolation: temporal, spatial, behavioural, and mechanical. In acoustic species such as anurans and orthopteran insects, the behavioural mechanisms involving species-specific advertisement calls and female phonotaxis toward conspecific calls are well established (Gerhardt and Huber 2002). However, the effectiveness of a behavioural isolating mechanism may be compromised between sympatric species when male advertisement signals and female preferences are not completely isolated (Pfennig 2000), or when syntopic species occur in close proximity in a multi-species acoustic lek (Bee

2014).

Examples of incomplete reproductive isolation are numerous in anurans, including *Bombina bombina* and *B. variegata* in Eastern Europe (Gollmann et al. 1988; MacCallum et al. 1998), the *Lithobates pipiens* complex in the South Western USA (Frost and Platz 1983), *Hyla cinerea* and *H. gratiosa* in eastern North America (Gerhardt 1974), and the *Litoria ewingi* complex in Australia (Watson et al. 1985; Littlejohn and Watson 1985; see also Littlejohn 1999). Furthermore for anurans, intra- and inter-specific interactions in multi-species leks are widespread (Littlejohn 1977; Gerhardt and Schwartz 1995). Due to the nature of incomplete reproductive isolation and interactions between sympatric species in close proximity, the risk of heterospecific mating is substantial in some anurans. For example, a population of *H. cinerea* and *H. gratiosa* exhibited evidence of 46.5 % of individuals with mixed parentage (Lamb and Avise 1986). The studies of heterospecific interaction in a multi-species lek often focus on the species pairs with incomplete reproductive isolation, thus the full extent of heterospecific interaction is difficult to assess. For instance, sympatric species with distinct advertisement calls are less ideal for studies of isolating mechanisms, although these species can still vocally interact.

When a behavioural isolating mechanism is not sufficient for reproductive isolation between syntopic species, additional mechanisms

are required to maintain species boundaries. Calling frogs may also have temporal and/or spatial isolating mechanisms by means of niche differentiation, to minimize interaction with syntopic species. Compared to other isolating mechanisms, temporal and/or spatial isolating mechanisms may readily take place, because no additional morphological or behavioural traits need to evolve. For instance, in a multi-species lek involving 12 anuran species, no overlap in the calling sites was reported between species that are able to hybridize (*i.e. H. cinerea* and *H. gratiosa*), whereas calling sites were shared between species pairs without hybridization abilities (*i.e. Acris gryllus*, *Rana clamitans* and *H. crucifer*, Etges 1987). Sympatric species may use strategies to prevent erroneous mating, either spatially, such as distinct microhabitat use between *D. japonicus* and *Pelophylax porosa brevipoda* (Naito et al. 2013), or temporally, such as three hylid frog communities with a similar temporal breeding season shifting their attendance to breeding sites when in sympatry (Duellman and Pyles 1983). Thus, the role of niche differentiation, which is traditionally related to the lessening of competition over food and/or shelters, may also be extended to reproductive isolation.

Two treefrog species occur on the Korean peninsula, *D. suweonensis* and *D. japonicus* (Jang et al. 2011; Roh et al. 2014). *Dryophytes japonicus* is widespread across East Asia, ranging from

Mongolia to Japan and from Manchuria to southern China (IUCN 2014b). In Korea, *D. japonicus* is abundant, even in seemingly inhospitable environments, such as high altitude mountain ranges, coastal pools, and rice paddies within urban settings (Jang et al. 2011; Roh et al. 2014). Conversely, *D. suweonensis* is an endangered species (Ministry of Environment of the Republic of Korea 2012; IUCN 2014a) restricted to coastal plains, almost exclusively in the northwest of the Republic of Korea (Kim et al. 2012b; Roh et al. 2014). *Dryophytes suweonensis* is sympatric with *D. japonicus* on the totality of its range. Almost everywhere male *D. suweonensis* are heard calling, male *D. japonicus* are also producing advertisement calls. These two species are morphologically similar, albeit not identical (Borzée et al. 2013), and exhibit a microhabitat differentiation for their calling location in rice paddies. *Dryophytes suweonensis* calls from the centre of rice paddies while *D. japonicus* calls from the edges, with an average separation of 8.14 m (Borzée *et al*. 2013, unpublished data). In addition, advertisement calls of these two treefrog species are distinct: *D. japonicus* calls are delivered faster in note repetition and lower in dominant frequency compared to *D. suweonensis* calls (Jang et al. 2011; Park et al. 2013).

Despite the differentiation in morphology, calling location, and advertisement call, it remains unclear how these two species are reproductively isolated from each other, as they can hybridize in a

laboratory setting (Kuramoto 1984), and are hybridizing over the whole range of *D. suweonensis* (unpublished data). To understand the isolating mechanisms for maintaining species boundaries, we investigated the diel movements of these species during the breeding season. We tracked individuals of both species using a harmonic detection finder (HDF) in order to investigate patterns of fine-scale movements for temporal and spatial differentiation in microhabitat use as an additional isolating mechanism. The results of this study are particularly relevant in determining the causes of population decline in *D. suweonensis*.

MATERIALS AND METHODS

Study populations and field localities

This study was conducted at four sites in the city of Paju (37.8862 °N, 126.7437 °E), Gyeonggi Province, close to the demilitarized zone (DMZ) between the Republic of Korea and the Democratic People's Republic of Korea. The study sites consisted of low-elevation rice paddies, representative of the habitat of *D. suweonensis* (Borzée and Jang 2015). The proximity to the DMZ has slowed economic development, allowing the study sites to contain one of the largest remaining populations of *D. suweonensis*. The four study sites were at least 200 m apart to prevent recapture, as male treefrogs are philopatric to their calling location during the breeding season (Berven and Grudzien 1990). This

study was conducted between June 17th and July 3rd, 2013, in the middle of the breeding season (Fujioka and Lane 1997; Roh et al. 2014). Species identification was based on differences in call characteristics (Park et al. 2013).

Harmonic detection finding

To track treefrogs, we used a HDF (model R2; RECCO AB; Lidingö, Sweden), which relied on radio wave reflectors that bounce signal waves back to the emitter (de Moura Presa et al. 2005; Leskovar and Sinsch 2005; Rowley and Alford 2007). This method has the advantage of being usable even on the smallest amphibians: *H. arborea* (Pellet et al. 2006), *Allobates femoralis* (Pašukonis et al. 2014a; Pašukonis et al. 2014bb), and *Litoria rheocola*, (Gourret et al. 2011). The use of HDF requires an antenna with a size tailored to each targeted individual. We soldered a Schottky diode (model R2; RECCO AB; Lidingö, Sweden) on a tin-plated copper wire to maintain the electric properties of the diode and the mechanical elasticity of the antennae. At one end, the antennae formed a loop to serve as an electric dipole, reflecting the directional and polarized microwaves emitted by the HDF. The Schottky diode was soldered in a fashion that creates a bridge between the two legs of the wire, which was used as the antenna, bent at 180º. To insulate the electrical dipole, the antennae were uniformly coated with a silicone spray

(model S-830 UL94 V-0; Nabakem; Seoul, Republic of Korea). We selected an initial antennae with two 8-cm legs, resulting in a 25-m effective range (Appendix 1). Upon reception of the reflected microwaves, the HDF translates it into an acoustic signal of varying intensity in function of the direction and distance to the dipole, thus allowing for the localization of the organism bearing the antenna (for details see Pellet et al. 2006). The range of the HDF drastically decreased when the electric dipole was underwater. For instance, the two-legged antenna with a 25-m range in air decreased in detection range to 3 m when not insulated, and 9 m when insulated under 3 cm of water (Annex 5.1).

The antennae were affixed to a waistband made out of gauze without cotton lining, for quick degradation in the field (Fukuyama et al. 1988; Hodgkison and Hero 2001; Pellet et al. 2006). The waistband weighed on average 0.05 g, and the antennae were 0.13 g (± 0.02). Each waistband with an antenna was prepared separately, with varying lengths for each individual, in order to match the antenna weight within 5 to 8 % (averaged at 6.3%) of the individual body mass (Hodgkison and Hero 2001).

Field tracking

The sample sizes were 39 male *D. japonicus* and 6 male *D. suweonensis,* in accordance with the permit from the Ministry of Environment of the Republic of Korea (permit 2013-16), as well as ethical

standards, as less than 300 calling males were recorded in the wild in 2012 (Kim et al. 2012b). We were not as successful at tracking female treefrogs (three *D. japonicus* and one *D. suweonensis*) as males, probably because females were often submerged, limiting the range of the HDF. Furthermore, there were not many female treefrogs available for tracking during the study period.

Following capture, each frog was fitted with the custom-made waistband and antenna on site and released at the capture point within 6 h. The HDF was then used to locate individuals every 20 min over a maximum of 72 h, with no elapsed time between inspections greater than 3 h, and an average interval of 65.99 ± 113.30 min (mean ± SD). Frogs were visually inspected every 6 h to prevent any adverse effects of the antennae on frog health (*i.e.* physical blockage). We included the tracking data for statistical analyses only when a frog was tracked for at least 18 h including one overnight calling period, to ensure that the individual was not displaying any anomalous behaviour. We removed the antennae from two *D. japonicus* individuals before completion of the experiment due to swollen legs. Of these two, only one individual was included in the analyses, because it had been tracked for more than 18 h.

After locating an individual, we recorded the season, time of day, distance travelled from the previous location (further referred to as "displacement"), height at which the individual was found, air

temperature, humidity (HT-350; Iondo; Seoul, Republic of Korea) and luminosity (Lux/Fc Light meter TM-201; Tenmars; Taipei, Taiwan). The season was the number of days since April 16th, 2013, when the earliest advertisement calls of both species were heard in our study area. Abiotic variables were taken at approximately 30 cm from the treefrog without disturbance. Displacement was defined as the distance moved between two consecutive HDF inspections. In addition, we calculated the displacement speed for each individual, which was the displacement divided by the time interval.

We noted the microhabitat in which individuals were found at each inspection, classifying them into one of five types: rice paddy (wetland where rice seedlings grew), ground (bare ground where vegetation did not grow), grass (ground with any kind of vegetation < 1 m high), bush (ground with any kind of vegetation > 1 m high), and buried (found under a substrate, at any depth). For the buried microhabitat, height was arbitrarily coded as "-1."

Lastly, we recorded any behaviour displayed by the frogs during tracking: calling (producing advertisement calls), encounter (when two frogs were less than 10 cm away from each other, without being amplexed), resting (all limbs in contact with the body, parallel and in contact with the substrate) to prevent water loss, mating (male and female amplexed), and feeding (catching or eating prey). The sample size was not

constant across behavioural variables, as field conditions prevented the recording of some variables (*e.g.* underwater or too high).

Chorus

We recorded the treefrog chorus in the field to determine the possibility of temporal isolation of calling activities between the two treefrog species. The calling activity of the two species was recorded at six localities in Gyeonggi Province, distinct from the field tracking sites. The six recording localities were previously known to harbour the two treefrog species. Recordings were conducted between May 16th and July 11th, 2013 with digital Flash Voice Recorders (model #: ICD-UX523; Sony Inc.; Tokyo, Japan) set with two stereo microphones 3 m apart at ground level. The recordings were started before 4 pm and stopped after dawn the next day around 10 am, allowing for a continuous recording of the chorusing behaviour. For each recording, we determined the time at which the advertisement calls of each species were first heard.

Statistics

We explored the possibility of microhabitat differentiation between *D. suweonensis* and *D. japonicus* using a *G*-test to determine the relationship between species and microhabitat. An individual was counted only once for each microhabitat. Next, we used a Generalized Linear Model to determine the factors important for the occurrence of treefrogs in each microhabitat. We tabulated the use of microhabitats for each of the

data points collected for an individual. The probability function for a microhabitat was multinomial with cumulative logit as the link function. The subsequent univariate analyses of the General Linear Model were performed independently for each species. The predictor variable was individual, and the covariates were season, time of day, temperature, and height. Because temperature, humidity, and luminosity were all highly correlated with each other (Pearson correlation > 0.801, $p < 0.001$), we used only temperature as the representative abiotic factor for all analyses.

To assess the extent of temporal and spatial separation for reproductive isolation between the two species, we calculated entry time, exit time, and distance to the bank when treefrogs used the rice paddy microhabitat. Differences between the two species for these variables were tested using independent *t*-tests. In the case of several occurrences for a movement to or from a microhabitat, the data were averaged for each individual. Two individuals were excluded from the dataset due to anomalous displacement, as they had been carried away while in amplexus, and another individual due to the early stop of tracking because of swollen legs.

A General Linear Model was used to study the displacement of the two species during tracking. The predictor variables were species, individual, and microhabitat, and the covariates were season, time of day, temperature, height, and time interval. Individual was nested within

species. We only used the phase of movement to calculate the displacement speed, excluding the phase of resting. We employed a likelihood ratio test to determine the differences between *D. suweonensis* and *D. japonicus* for calling and resting, in function of the microhabitats.

We conducted first-order Markov chain analyses to test for the existence of non-random temporal associations between movement patterns among the five microhabitats, in the two species. We tabulated a 5 × 5 transition matrix for both species by counting all instances in which one microhabitat led to a succeeding microhabitat in consecutive observations. We computed the expected frequency of a particular transition by dividing the subtotal of the transition, 25, and subsequently used a chi-square goodness-of-fit test to determine the significance of the transition.

The analysis of the chorusing data was conducted through a Chi-square test of independence to determine the overlap in the calling activity of the two species. All statistical analyses were computed through SPSS (ver. 20.0, SPSS, Inc., Chicago, IL, USA).

RESULTS

Microhabitat

Males of both *D. japonicus* (n_{Hj} = 32) and *D. suweonensis* (n_{Hs} = 6) occurred in all five microhabitats in the present study (Fig. 5.1).

Species and microhabitat were not independent of each other (Likelihood ratio = 43.434, *df* = 4, *P* < 0.001). *Dryophytes suweonensis* was often found in the rice paddy, ground, and grass microhabitats, while *D. japonicus* was frequently associated with the grass and bush microhabitats. The results of the Generalized Linear Model with multinomial distribution for microhabitat was significant for *D. japonicus* (Likelihood ratio chi-square = 2723.83, *df* = 372, *P* < 0.001) and for *D. suweonensis* (Likelihood ratio chi-square = 384.74, *df* = 92, *P* < 0.001).

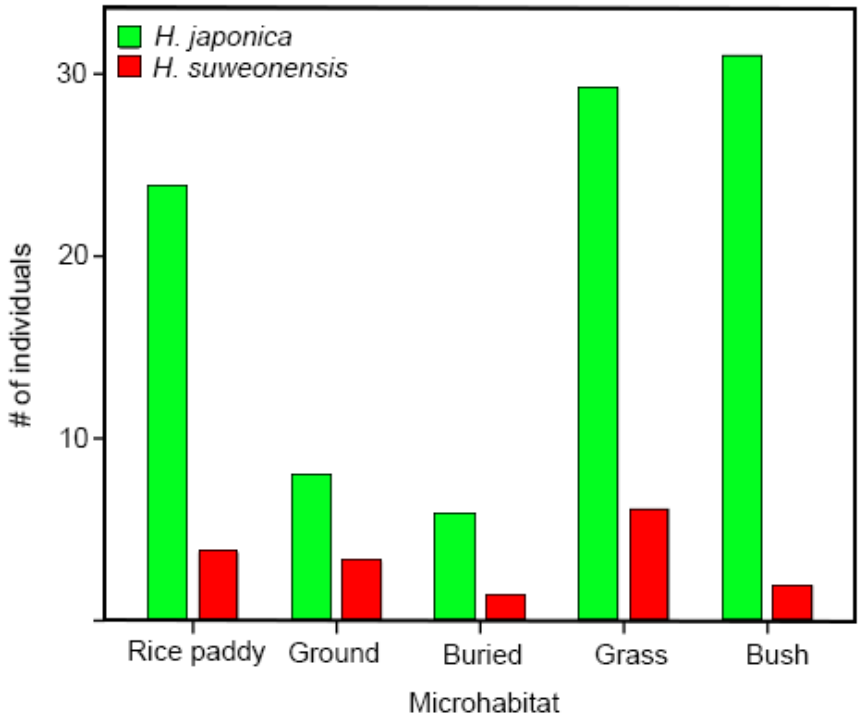

Figure 5.1. Use of five microhabitat types by male *Dryophytes japonicus* (green, n_{Hj} = 32) and *D. suweonensis* (red, n_{Hs} = 6) during the breeding season. The y axis represents the number of individuals. One individual was counted only once for each microhabitat type if it was found there during tracking. The sample sizes differed for each microhabitat as not all individuals visited all microhabitats.

For *D. japonicus*, all predictor variables were significant, individual nested within species (Wald chi-square = 218.16, $df = 24$, $P < 0.001$), season (Wald chi-square = 37.87, $df = 14$, $P < 0.001$), time of day (Wald chi-square = 304.66, $df = 167$, $P < 0.026$), temperature (Wald chi-square = 208.62, $df = 106$, $P < 0.001$) and height (Wald chi-square = 4358.70, $df = 33$, $P < 0.001$). For *D. suweonensis,* individual nested within species (Wald chi-square = 88.91, $df = 4$, $P < 0.001$), season (Wald chi-square = 676.24, $df = 1$, $P < 0.001$), time of day (Wald chi-square = 117.51, $df = 28$, $P < 0.001$) and height (Wald chi-square = 264.31, $df = 6$, $P < 0.001$) were significant, but temperature (Wald chi-square = 7.07, $df = 4$, $P = 0.132$) was not.

The subsequent univariate analyses showed that most predictor variables were significant in each microhabitat for *D. japonicus* and *D. suweonensis* (Table 5.1). Height was statistically significant for both species in all microhabitats.

In all five microhabitats, time of day was earlier for *D. suweonensis* than for *D. japonicus* (Table 5.2). That is, male *D. suweonensis* were more likely to be found earlier in all microhabitats than male *D. japonicus*. Time of entry was significantly different between the two species for rice paddy, grass, and bush, but was not significantly different for ground and buried (Table 5.2). Male *D. suweonensis* moved 3:05 h, 4:27 h, and 1:34 h earlier than male *D. japonicus* into rice paddy,

grass, and bush, respectively.

Time of exit was significantly different between the two species for rice paddy (0:42 h) and bush (6:10 h); *D. suweonensis* moved out of the bush 1:10 h before the entry of *D. japonicus*. Thus, the diel rhythm provided time periods of no heterospecific interference for male *D. suweonensis,* especially in rice paddy and bush by moving in advance of male of *D. japonicus* (Table 5.2). Male *D. suweonensis* (6:33 ± 1:51 h, mean ± SD, n_{Hs} = 5) spent significantly more time in rice paddy than *D. japonicus* (3:49 ±, 1:59 min, mean ± SD, n_{Hj} = 18) (*t* test; $t = 2.35$, $df = 21$, $P = 0.025$). Durations of stay were not significantly different between the two species for the other microhabitats (*t* test; $t \leq 1.09$, $df \geq 30$, $P \geq 0.288$). *Dryophytes suweonensis* males tended to stay at or near ground level in all five microhabitats, whereas male *D. japonicus* climbed high on bush (Fig. 5.2). For the bush microhabitat, male *D. suweonensis* were found 19.16 ± 6.49 cm high (mean ± SD, n_{Hs} = 6). In contrast, male *D. japonicus* were found on average 124.23 ± 103.51 cm high (n_{Hj} = 17) when resting on bush.

Furthermore, when in rice paddies, the distance to the bank was significantly different between *D. japonicus* (70.61 ± 88.97 cm; n_{Hj} = 20) and *D. suweonensis* (650.00 ± 160.45 cm; n_{Hs} = 5) (*t*-test; $t = 12.92$, $df = 23$, $P > 0.001$).

Temperature was not a significant factor for the General Linear

Model, probably because it was not one of the factors eliciting a biological response, or because time of day and temperature were correlated with each other (Pearson correlation coefficient; $r = 0.19$, $P < 0.001$, $n = 1141$). However, we present the distributions of temperature over the five microhabitats because temperature may be critical for understanding ecological differences in niche specialization for the two species. *Dryophytes suweonensis* was found in the rice paddy, ground, and buried microhabitats at higher temperatures than *D. japonicus*, with an opposite pattern for the grass microhabitat (Fig. 5.3).

Because ground was a microhabitat of transition between rice paddy and resting places, and because *D. suweonensis* moved across microhabitats earlier than *D. japonicus*, the temperatures at which *D. suweonensis* individuals were found while moving were generally higher than those for *D. japonicus*. At higher temperatures, also representative of a time of day matching with the afternoon, male *D. suweonensis* moved across different microhabitats to reach rice paddies for calling. In contrast, male *D. japonicus* moved across microhabitats towards the calling sites later than *D. suweonensis,* when temperatures were lower, and thus spent the warmest part of the day in the grass and bush microhabitats. In summary, the variation in time of entry and exit from microhabitats was species specific and may be correlated with abiotic factors including time of day.

Table 5.1. Results of the Generalized Linear Model for microhabitat in *Dryophytes japonicus* and *D. suweonensis*. The microhabitats in which treefrogs were found were noted along with abiotic factors. Sample sizes are variables, depending on species and microhabitat.

		Dryophytes japonicus				*Dryophytes suweonensis*			
		df	χ^2	*F*	*P*	*df*	χ^2	*F*	*P*
Rice paddy	Season	1	0.05	0.37	0.541	1	0.03	0.26	0.613
	Time of day	1	19.94	160.97	< 0.001	1	6.59	57.81	**< 0.001**
	Temperature	1	21.75	175.59	< 0.001	1	4.07	35.7	**< 0.001**
	Height	1	15.64	126.23	< 0.001	1	3.04	26.64	**< 0.001**
	Individual	1	0.62	5.02	0.025	1	0.02	0.2	0.653
	Error	972	0.12			157	0.11		
Ground	Season	1	0.01	0.08	0.773	1	1.77	14.99	**< 0.001**
	Time of day	1	0	0.01	0.927	1	1.86	15.71	**< 0.001**
	Temperature	1	0.01	0.16	0.687	1	0.36	3.06	0.082
	Height	1	1.83	25.17	< 0.001	1	2	16.94	**< 0.001**
	Individual	1	0.1	1.4	0.236	1	1.91	16.18	**< 0.001**
	Error	972	0.07			157	0.12		
Buried	Season	1	0.09	1.72	0.19	1	0.7	10.54	**0.001**
	Time of day	1	0.01	0.14	0.708	1	0.11	1.62	0.205
	Temperature	1	1.71	32.59	< 0.001	1	0.59	8.85	**0.003**
	Height	1	1.51	28.79	< 0.001	1	0.61	9.2	**0.003**
	Individual	1	0.23	4.39	0.036	1	0.55	8.2	**0.005**
	Error	972	0.05			157	0.07		
Grass	Season	1	2	11.16	0.001	1	0.72	5.51	**0.002**
	Time of day	1	11.68	65.24	< 0.001	1	0.35	2.65	0.105
	Temperature	1	9.61	53.68	< 0.001	1	0.02	0.18	0.674
	Height	1	2.39	13.33	< 0.001	1	10.8	82.85	**< 0.001**
	Individual	1	0.04	0.23	0.63	1	0.34	2.59	0.109
	Error	972	0.18			157	0.13		
Bush	Season	1	2.02	16.46	< 0.001	1	0.28	3.33	0.07
	Time of day	1	0.97	7.95	0.005	1	0.89	10.77	**0.001**
	Temperature	1	0.13	1.1	0.295	1	0.25	2.97	0.087
	Height	1	65.28	532.99	< 0.001	1	0.43	5.19	0.024
	Individual	1	0.55	4.52	0.034	1	0.01	0.1	0.757
	Error	972	0.12			157	0.08		

Displacement

Displacement speed was significantly higher in male *D. suweonensis* (5.61 ± 9.59 cm/min, mean ± SD; n_{Hs} = 6) than in male *D. japonicus* (2.30 ± 6.46 cm/min; n_{Hj} = 26), based on an analysis including only the periods of movement and excluding periods of resting (independent samples *t* test, equal variances not assumed; t = -2.84, df = 9.86, P = 0.018). One *D. japonicus* male showed the highest displacement speed of all tracked males, but this male was carried away by a female treefrog while in amplexus.

The results of the General Linear Model for displacement speed (n_{Hj} = 25, n_{Hs} = 6) revealed that species, individual, microhabitat, time of day, height, and interval were significant, but season and temperature were not (Table 5.3). The displacement speeds were generally higher at times between midnight and dawn for both treefrog species.

Dryophytes japonicus (n_{Hj} = 25) displacement speed was below 10 cm/min from 5.30 am until midnight, however the displacement speed exceeded 10 cm/min between midnight and 5 am (Fig. 5.4). Likewise for *D. suweonensis* (n_{Hs} = 6), the displacement speed was over 20 cm/min between midnight and 5 am (Fig. 5.4).

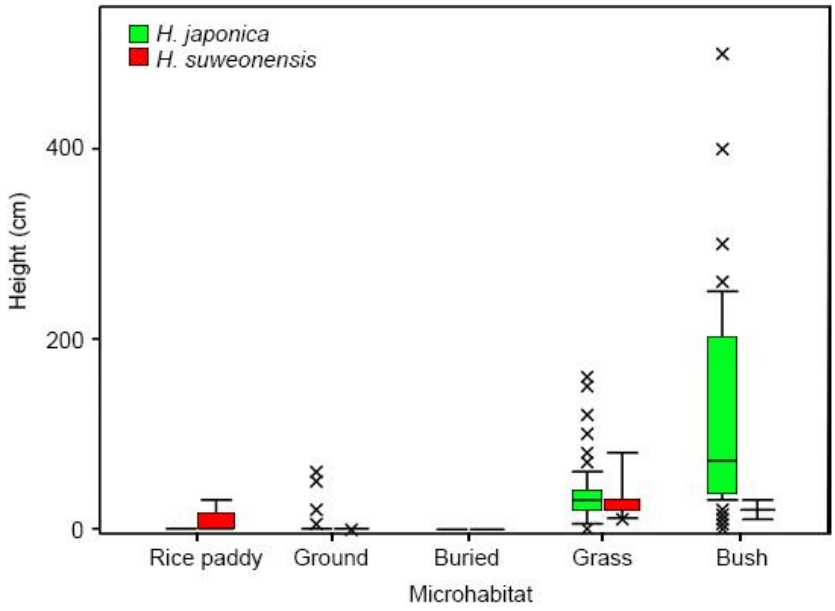

Figure 5.2. Boxplots of the height in the five microhabitat types in *Dryophytes japonicus* (green) and *D. suweonensis* (red) during the breeding season. X denotes outliers. This analysis was based on 17 *D. japonicus* and 6 *D. suweonensis*.

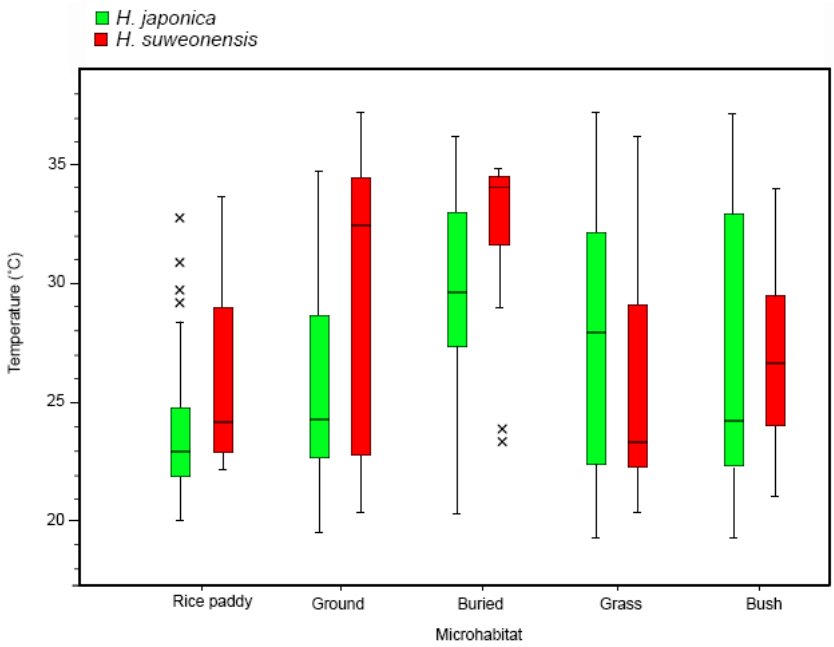

Figure 5.3. Boxplots of the temperature across the five microhabitat types in *Dryophytes japonicus* (green) and *D. suweonensis* (red) during the breeding season. X denotes outliers. This analysis was based on all individuals: 32 male *D. japonicus* and 6 male *D. suweonensis*.

Table 5.2. Times of entry and exit for the five microhabitats in *Dryophytes suweonensis* and *D. japonicus*. The lack of consistency between sample sizes for entry and exit time is due to the exclusion of the last single observation for moving out of a microhabitat, if it was the last one visited by an individual prior to release. Sample sizes are also variables depending on species and microhabitat: rice paddy ($n_{Hj\ obs}$ = 238, $n_{Hs\ obs}$ = 37), ground ($n_{Hj\ obs}$ = 80, $n_{Hs\ obs}$ = 32), buried ($n_{Hj\ obs}$ = 58,$n_{Hs\ obs}$ = 14), grass ($n_{Hj\ obs}$ = 292, $n_{Hs\ obs}$ = 61), and bush ($n_{Hj\ obs}$ = 310, $n_{Hs\ obs}$ = 19). Time is in the format (HH:MM).

	Dryophytes suweonensis				*Dryophytes japonicus*				*t* test		
	Avr	SD	N ind	N obs	Avr	SD	N ind	N obs	*t*	*df*	*p*
Rice paddy	16:01	07:04	5	7	19:06	04:11	20	44	4.14	24	<0.001
Ground	13:22	12:53	5	7	15:23	10:15	18	27	1.11	21	0.279
Buried	13:33	04:11	2	3	15:13	10:33	8	8	0.36	8	0.727
Grass	04:03	03:11	4	6	12:29	08:31	24	43	3.45	26	0.005
Bush	06:10	02:46	3	4	11:55	07:44	14	22	2.86	15	0.012
	Time of exit										
	Dryophytes suweonensis				*Dryophytes japonicus*				*t* test		
	Avr	SD	N ind	N obs	Avr	SD	N ind	N obs	*t*	*df*	*p*
Rice paddy	22:39	00:29	5	7	23:21	02:30	19	44	2.37	23	0.027
Ground	11:04	00:25	5	7	14:49	00:24	18	27	1.2	21	0.242
Buried	15:41	00:05	2	3	16:25	00:16	8	8	0.21	8	0.836
Grass	15:22	00:13	3	6	15:32	00:22	24	43	0.33	25	0.742
Bush	10:45	00:06	4	4	16:55	00:22	14	22	2.19	16	0.043

* SD is the standard deviation, avr is average, ind is individual and obs is observed.

Analyses of the first-order Markov chain transition showed a non-random association between movement patterns in the two species (likelihood ratio test: Λ =1487.831, df = 16, $P < 0.001$, n = 951 movements for *D. japonicus*; Λ =240.757, df = 16, $P < 0.001$ n = 157 movements for *D. suweonensis*). However, the movement patterns of the two species were not different from each other (Mantel-Haeznel common odds ratio = ln(estimate) = 0.332, P = 0.172). Thus, we pooled the movement data of both species.

The analyses of the Markov chain showed that a higher percentage of movements occurred between rice paddy and ground, ground and grass, and rice paddy and grass. However, individuals tended to stay in the grass and bush microhabitats once they were there (Fig. 5.5). Thus, male treefrogs seemed to stay in the rice paddy microhabitat for calling, and in the grass or bush microhabitats for resting.

Behaviour

All five behaviours were observed for *D. japonicus*, but only calling and resting were documented for *D. suweonensis*, likely due to the small sample size. Thus, we removed encounter, mating, and feeding behaviours (respectively 5, 4 and 3 occurrences for the two species combined) from further analyses.

Table 5.3. General linear model for displacement speed (n_{Hj} = 25, n_{Hs} = 6) and seven covariates.

	df	Mean Square	*F*	*P*
Species	1	224706	25.412	< 0.001
Individual	30	30285	3.425	< 0.001
Microhabitat	4	67969	7.687	< 0.001
Season	1	5422	0.613	0.434
Time of day	1	159162	18.00	< 0.001
Temperature	1	8945	1.012	0.315
Height	1	150173	16.983	< 0.001
Time interval	1	207977	23.52	< 0.001
Error	1097	8843		

The two treefrog species were significantly different in their choices of microhabitats when resting (Likelihood ratio = 28.25; $df = 3$, $P < 0.001$; $n_{Hj} = 10$, $n_{Hs} = 4$), but not when calling (Likelihood ratio = 1.387; $df = 2$, $P = 0.500$; $n_{Hj} = 19$, $n_{Hs} = 4$). When resting, male *D. japonicus* were found in the grass or bush microhabitats, whereas male *D. suweonensis* were typically found in grass (Fig. 5.6).

Chorus

In all six field monitorings, *D. suweonensis* initiated calling ahead of *D. japonicus*. The start time of the advertisement calls of *D. suweonensis* ranged from 16:05 to 19:47, and those of *D. japonicus* ranged from 18:17 to 19:43. *D. suweonensis* started calling 102.33 ± 71.64 min (mean ± SD; range: 27 – 228 min, $n = 696$) earlier than *D. japonicus* (*t* test; $t = 3.49$, $df = 5$, $P = 0.017$).

DISCUSSION

The results of our field tracking revealed that males of the two species, *Dryophytes suweonensis* and *D. japonicus*, occurred in all five microhabitats identified in this study during the breeding season (Fig. 5.1). Furthermore, the movement patterns of both treefrog species were similar across the microhabitats (Fig. 5.5). Despite the similarities, we found that the two species differed in temporal and spatial patterns of microhabitat use during the breeding season.

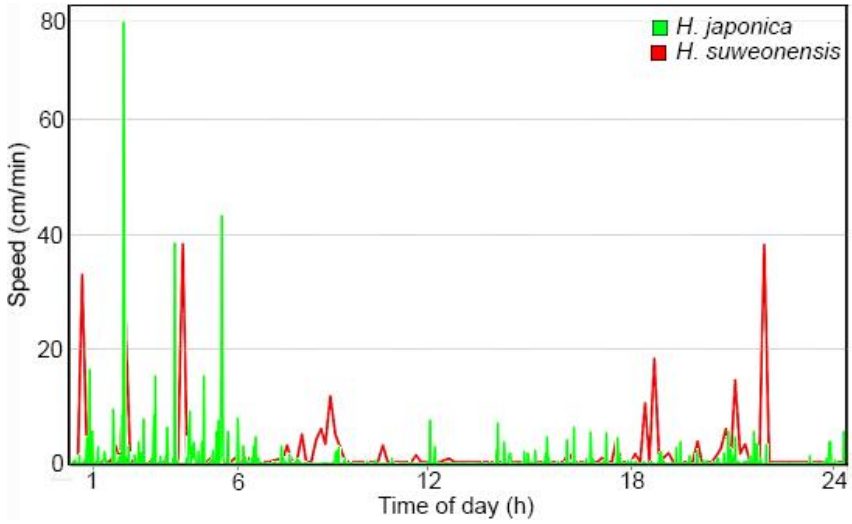

Figure 5.4. Displacement speed (cm/min) as a function of time of day for *Dryophytes japonicus* (green, n_{Hj} = 32) and *D. suweonensis* (red, n_{Hs} = 6). Displacement speed was calculated as (distance travelled)/(time period).

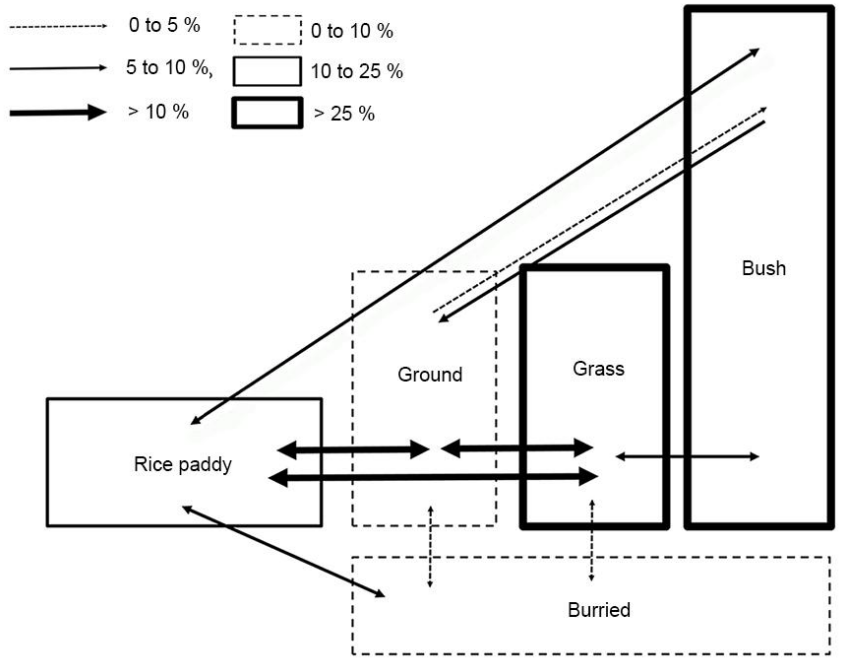

Figure 5.5. Markov-chain diagram for diel movements of male *Dryophytes japonicus* and *D. suweonensis* (combined) across microhabitats during the breeding season. Rectangles denote repetition of the same behaviour. Arrows are the transition from one behaviour (arrow tail) to the next (arrow head), with thickness representing the probability of the transition. This analysis was based on all 951 *D. japonicus* and 157 *D. suweonensis* movements.

First, there was a spatial separation in calling location between the two species in rice paddies (Fig. 5.6). Second, the time of entry into rice paddies was on average 3 h earlier in *D. suweonensis* than in *D. japonicus*.

The early entry into the rice paddies created a time interval during which male *D. suweonensis* produced advertisement calls alone, with more than 2 h of non-overlap in call production during which non-erroneous mating could occur for *D. suweonensis* (Table 5.2). In the independent call monitoring, *D. suweonensis* always called earlier than *D. japonicus*. Third, *D. suweonensis* tended to rely more on grass than on bush when resting, and the reverse was true for *D. japonicus* (Fig. 5.6). Even if male *D. suweonensis* were found in the bush microhabitat, the two treefrog species were separated by height (Fig. 5.2). Thus, patterns of movements across microhabitats indicate a spatial and temporal segregation between the two species.

The temporal and spatial differentiations in microhabitat use seemed to explain the differences in biotic and abiotic factors of daily movements between the two species. *Dryophytes suweonensis* calls from the centre of the rice paddies and thus was required to travel further to reach its breeding site. Due to the difference in distance travelled from the resting places to the rice paddies, the displacement speed seemed to be higher in *D. suweonensis* than in *D. japonicus*. Male *D. suweonensis* generally moved earlier into microhabitats than *D. japonicus*.

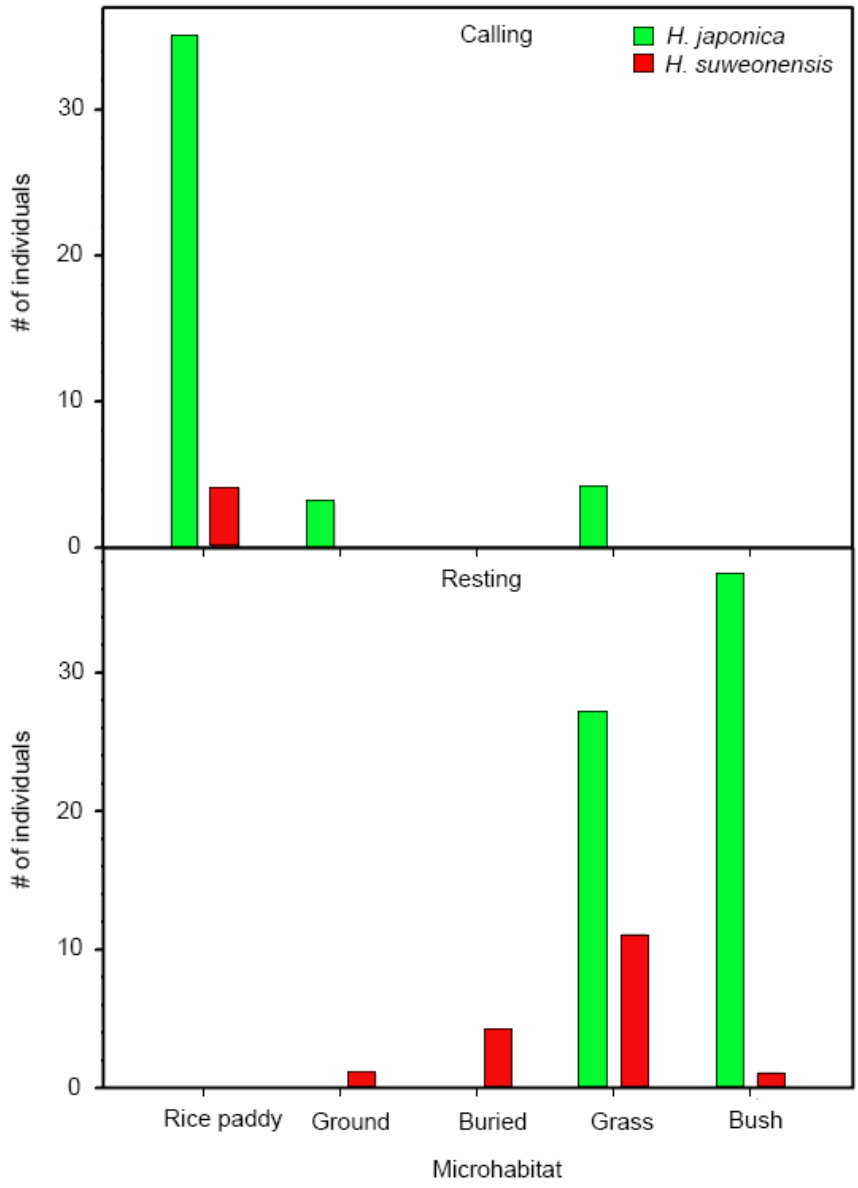

Figure 5.6. Number of individuals for calling and resting behaviours across the five microhabitat types for *Dryophytes japonicus* (green) and *D. suweonensis* (red). The two species were significantly different in their choices of microhabitats when resting (n_{Hj} = 10, n_{Hs} = 4), but not when calling (n_{Hj} = 19, n_{Hs} = 4).

Thus, temperatures at which treefrogs were found in each microhabitat were higher in *D. suweonensis* than in *D. japonicus*, although temperature itself may not be the factor leading to the initiation of movements across microhabitats by treefrogs.

The difference in the calling sites in rice paddies and the difference in time of entry may be effective for reproductive isolation between *D. suweonensis* and *D. japonicus*. Production of advertisement calls by *D. suweonensis* before the arrival of *D. japonicus* males in the rice paddies constitutes a temporal isolating mechanism, provided that female *D. suweonensis* also moved into the rice paddy microhabitat early. Although data for females in this study were scarce, the three female *D. japonicus* and the lone female *D. suweonensis* did show attendance to breeding choruses in rice paddies in our study. Female phonotaxis towards male chorus and amplexus at the location of calling is widespread in amphibians (Wells 1977), particularly in treefrogs (Fellers 1979a, b; Bevier 1997; Jaquiéry et al. 2010). Due to the commonalities in the ecology of Hylids (Moen et al. 2013), this generality may be extendable to Korean Hylids and may be applied to the four females of this study. Thus, the temporal and spatial differentiation in rice paddies may provide an additional layer of isolation mechanism between *D. suweonensis* and *D. japonicus*, in addition to the differences in advertisement calls.

The temporal and spatial differentiation in the grass and bush

microhabitats between *D. suweonensis* and *D. japonicus* during the breeding season may be a case of ecological specialization (Devictor et al. 2010; Poisot et al. 2011). Evolution of ecological specialization may arise to lessen competition over resource use (Graham et al. 2004; Duré and Kehr 2001). Male *D. suweonensis* are on average 5% smaller (snout-vent length) than male *D. japonicus* (Borzée et al. 2013). This size difference, as well as the differentiation in microhabitat use, could be a resulting correlate of the different ecologies and niche requirements. For instance, *D. suweonensis* may be a better competitor in the grass microhabitat than in the bush habitat, compared with *D. japonicus*. Conversely, the niche separation may be a result of the divergent evolution of the two species (Hua et al. 2009), spatially brought together in substitute breeding habitats (Borzée and Jang 2015) through the development of rice paddies (Liu et al. 2007; Fuller et al. 2007; Fuller et al. 2008). Under this scenario, the differentiation in microhabitat use is the result of restriction in the availability of environments without evolutionary change (Bolnick et al. 2003; Devictor et al. 2010).

We failed to detect any difference in the movement patterns across microhabitats between the two species (Fig. 5.5). This failure may stem from the small sample size for *D. suweonensis*. More likely, the failure of distinction in microhabitat use may reflect similar ecological requirements of the diel cycles during the breeding season for males of the two species.

Nocturnal male frogs typically alternate between locomotor and reproductive efforts during the night phase of the diel cycle (Oishi et al. 2004) and resting during the day phase. In treefrogs, resting characteristically occurs in vegetation or buried near the calling site (e.g. de Oliveira and Eterovick 2010).

Microhabitat selection, leading male *D. suweonensis* to rely on the grass microhabitat during resting (Fig. 5.6), makes these individuals especially vulnerable to disturbances such as the use of herbicide and mowing of grass and may be one of the factors leading to the endangered status of *D. suweonensis*. Large scale mowing can also be lethal through the destruction of the habitat of prey. Besides, the conservation of natural landscape structures such as edges and levees surrounding rice paddies will help in the conservation of *D. suweonensis*, due to the large amount of time spent in these microhabitats. These measures are urgent, as the remaining populations of *D. suweonensis* are extremely small and ecological knowledge for the species is conspicuously absent (Park *et al*. 2014). The conservation of these species would also benefit related organisms sharing the same ecosystem (Cody and Diamond 1975; Rutten et al. 2010; Eccard and Ylönen 2003), such as the endangered Golden Spotted Frog (*Pelophylax plancyi*) and the Narrow-mouthed Toad (*Kaoula borealis*).

Chapter six

ASYMMETRIC COMPETITION OVER CALLING SITES IN TWO CLOSELY RELATED TREEFROG SPECIES.

ABSTRACT

Interspecific competition occurs when one species using a resource limits the use of that resource by another species. A dominance relationship between the species competing over a resource may result in asymmetric competition. Here, we tested the hypothesis that two sympatric treefrog species, the endangered *Dryophytes suweonensis* and the abundant *D. japonicus*, compete with each other over calling sites. We observed the locations of calling individuals of the two treefrog species in rice paddies and tested whether removing one species affected the calling locations of the other species. Individuals of the two species were spatially isolated within rice paddies, with *D. japonicus* at the edges and *D. suweonensis* in the interior. Male *D. suweonensis* moved towards the edges of rice paddies when male *D. japonicus* were removed from the area, whereas male *D. japonicus* hardly moved when male *D. suweonensis* were removed. The results of both studies are consistent with asymmetric interspecific competition, in which the calling locations of *D. suweonensis* are affected by the calling activity of *D. japonicus*. In addition, *D. japonicus* were found "sitting" on the substrate during call production,

whereas *D. suweonensis* were "holding" onto vegetation. The difference in calling posture may represent an adaptive response to asymmetric interspecific competition.

INTRODUCTION

When two or more species share a common resource, the consumption of that resource by one of the species creates a "resource depletion zone" for the other species (*sensu* Schluter 2000). Such competition may lead to interspecific niche partitioning (Katzner et al. 2003). Competition is asymmetrical when a dominant species limits the other species' access to a resource. In amphibians, the salamander larvae *Ambystoma talpoideum* and *A. maculatum* compete with each other over food, and the latter species has a lower survival rate in the presence of the former (Walls and Jaeger 1987). Asymmetrical competition in the context of amphibian breeding ecology is well illustrated by green frogs (*Rana clamitans*) spawning closer to the edges of ponds when in the presence of bullfrogs (*R. catesbeiana;* Herrick 2013). In order to be subject to competition, two or more competing species must share a common resource, and resource use by one species should affect that of the other species, as this paper aims to exemplify in the framework of asymmetric competition in amphibian breeding assemblages.

The nature of interspecific competition is diverse in adult

amphibians and often occurs over food, habitat, or acoustic space used for signalling (Gerhardt and Huber 2002). Males of different species in a single location may defend their resources by using ritualised displays, such as calls (Reichert and Gerhardt 2013; Wells 1977; Wells and Schwartz 2007; Dyson et al. 2013), thus avoiding direct physical contact (Gerhardt and Huber 2002). The possible consequences of interspecific competition include temporal or spatial segregation between species, leading to niche partitioning (Schluter 2000; Campbell et al. 2006; Gerhardt 2013; Rohde 2006). A niche is defined as a localised microhabitat with biotic and abiotic variables that are different from those of the habitat as a whole. Niche segregation can be used as a tool to detect interspecific competition.

Two treefrog species are present in the Republic of Korea: *Dryophytes japonicus*, widespread throughout North East Asia, and *D. suweonensis*, present entirely within the distribution of *D. japonicus* on the western coastal plains of Korea. *Dryophytes suweonensis* is potentially synonymous to *H. immaculata* (Li et al. 2015), and it was recently suggested that its genus name be changed to *Dryophytes* (Duellman et al. 2016). The two species diverged between 3.2 and 7.1 million years ago (Yang and Park 1988a; Lee and Park 1992). Their advertisement calls are structurally similar, consisting of a train of single notes, but differ in call properties. The note repetition rate is slightly higher and the dominant

frequency slightly lower in *D. japonicus* than in *D. suweonensis*(Jang et al. 2011; Park et al. 2013). Distinguishing these two species based on morphology is difficult, although male *D. suweonensis* are more slender than male *D. japonicus* (Borzée et al. 2013). Both treefrog species are primarily found in rice paddies, as natural wetlands adequate for breeding are extremely rare (Borzée and Jang 2015). These two treefrog species are found together at the same time and location (Roh et al. 2014).

Male treefrogs typically produce advertisement calls on the ground, branches, or tree trunks. In a lone semi-natural habitat for *D. suweonensis*, males of both species were calling at the edge of the wetland (Borzée and Jang 2015). However, male *D. suweonensis* generally call in the middle of rice paddies and *D. japonicus* males call at the edge of rice paddies (Borzée et al. 2016a). The levee of rice paddies are typically composed of soils with grasses, which provide solid sites for calling treefrog males. During the breeding season, the middle of rice paddies is flooded and provides no solid ground for call production. Thus, it has been argued that the levee is an ideal location for producing advertisement calls, compared to other parts of a rice paddy. Furthermore, when female treefrogs move towards the rice paddy in order to mate and spawn, calling males located on a levee are likely to be the first to encounter females. Thus, male treefrogs prefer the levees of rice paddies when producing advertisement calls.

In the Republic of Korea, rice paddies are typically clustered together for ease of irrigation and management. A rice-paddy complex refers to a contiguous assemblage of paddies, which are divided by narrow banks, levees, or one-lane roads. Here, we used field observations and a removal experiment to investigate whether niche segregation occurs between *D. japonicus* and *D. suweonensis* in rice paddies. Niche segregation for calling sites was assessed within rice paddies and within rice-paddy complexes, where similar rice paddies are typically grouped together. Niche segregation at calling sites may simply reflect differential use of time and space in a rice paddy by the two treefrog species. However, competition between the two species may affect the calling locations of one or both of the sympatric species. Thus, we hypothesised that the removal of one of the species would be correlated with an expansion in the range of calling sites used by the other. At the rice-paddy complex scale, we expected commonalities in the distribution of the two species in terms of landscape use, owing to similarities in their general ecological requirements.

MATERIALS AND METHODS

Study area and species.

This research consisted of observational studies and a removal experiment. All experimental removals were approved and carried out in

accordance with the guidelines of the Ministry of Environment of the Republic of Korea (permit numbers 2015-03, 2015-05, 2015-6, and 2015-28). The observational studies were conducted in the city of Paju, Gyeonggi province, and the removal experiment was conducted throughout the range of *Dryophytes suweonensis* in the Republic of Korea (Fig. 6.1). Anuran species at the study sites generally included *Pelophylax nigromaculatus*, *P. chosenicus*, *D. japonicus*, and *D. suweonensis*. Adult *Rana coreana*, which are early spring breeders, were present, but they did not produce advertisement calls at our study sites during the study period. *D. japonicus* spawns in shallow water between late April and early July, while *D. suweonensis* breeds from May to late June. Therefore, observational studies and removal experiments were conducted between 17 June and 1 July 2013 and between 19 and 28 June 2015, respectively. Both observational and experimental studies took place between 19:00 and 02:30 the next day, matching the peak calling activity of *D. japonicus* (Yoo and Jang 2012). The rice-paddy complexes we investigated were separated from each other either by more than 2000 m or by non-crossable landscape elements (Ashley and Robinson 1996; Ray et al. 2002) and were therefore considered independent for statistical analyses (Roh et al. 2014).

Before the observational studies and removal experiments, we randomly selected rice paddies in rice-paddy complexes. To verify the

presence of calling males of both species, we spent 5 min assessing the calling activity in each rice paddy prior to the experiment. If no calling activity was registered, the protocol was repeated until advertisement calls were detected from either Hylid species. No form of aggression between the two species has been published, and the only antagonistic relationship is assumed to be through advertisement calls Kuramoto 1980; Jang et al. 2011; Park et al. 2013. All sampling was completed in rice paddies where treefrogs did not use floating plants or muddy patches above water level as substrates. During our study, the water depth was 20 cm on average, and the seedlings varied from 20 to 50 cm high. Patches above water level, later in the season, may affect the position of *D. japonicus* within rice paddies. The location of females during the breeding season is not known, and was inferred from that of other Hylid species.

Calling location within a rice paddy: observational study.

To determine the niche segregation for calling location within a rice paddy, we conducted acoustic monitoring of the two treefrog species in 16 rice paddies that were randomly sampled from 6 rice-paddy complexes (Figure 6.1).

Each paddy in a complex was given a unique number, and rice paddies were randomly chosen for acoustic monitoring (Brandao Apps, 2010). The minimum distance between adjacent rice paddies was 85 m. Adjacent rice paddies less than 100 m apart were not sampled on the same

night.

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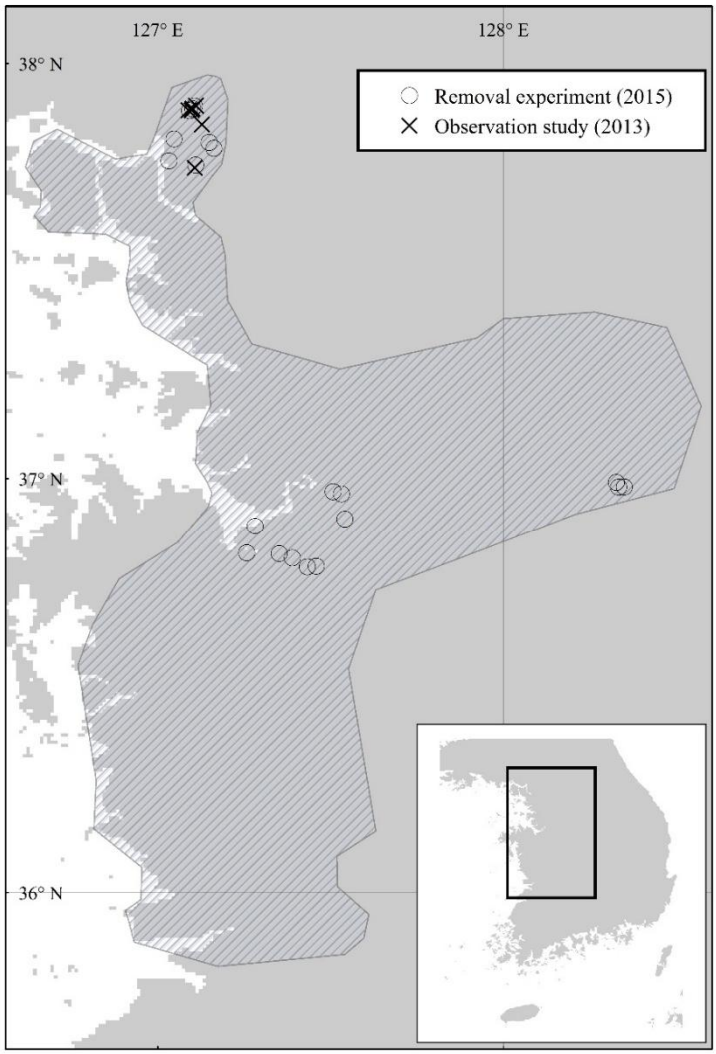


Figure 6.1. Map showing the range of *Dryophytes suweonensis* and the sampling sites used in this study. The species range (dashed area) is limited in the east by the elevation of the Taebaek mountain range and to the west by the Yellow Sea. *D. japonicus* is present on all landmasses visible on the map. This map was generated with ArcMap 9.3 (Environmental Systems Resource Institute, Redlands, USA; http://www.esri.com/).

Once a rice paddy was selected, LED markers (SY-MN-02; Xiamen Shangyi Technologies Co. Ltd.; Xiamen Fujian, China) were silently deployed on two adjacent banks of the rice paddy every three or five meters, with a maximum of 20 LED markers per rice paddy to prevent disturbance The intensity of each LED marker was set at 3.09 (± 0.53) lux at 1 m.

The effect of the LEDs on chorus dynamics was investigated before and after recording, and the number of individuals calling did not differ significantly depending on the presence of the markers (two-sided *t* test; $t = 0.194$, $df = 15$, $P = 0.849$). Following the setting of these markers, a Sony PCM D-50 recorder fitted with a directional microphone (Sennheiser ME62+K6 powering module, frequency response: 20-20000 Hz ±2.5 dB; Wedermark, Germany) covered with a wind screen (MZW20-1 Blimp Windscreen; Sennhwiser; Wedermark, Germany) was used to record along two continuous banks, by pointing towards the opposite bank. The microphone position relative to the markers was recorded to provide evidence for calling activity at a given location in the rice paddy. In addition, two people noted the locations at which frogs were calling on previously prepared maps of the rice paddy. Each person was assigned a separate side of the rice paddy, and the distance from the bank to the location of the calling individual was assessed using the LED markers. The minimum listening period was five min, with minor variations owing

to the time needed to walk around the rice paddy. The results obtained from the two maps and the recordings were compared, and only matching data were used for further analysis.

After the observations, we noted the physical characteristics of the rice paddies, including their surfaces. The presence of the following characteristics was also noted for each rice paddy: vegetation, one-lane roads, and ditches. Vegetation had to be at least 30 cm thick and high to be recorded. A one-lane road was defined as a paved road used for agricultural vehicles, with infrequent automobile use. Ditches were used for irrigation, and a rice paddy was in contact with a ditch when surrounded by other rice paddies. Concrete ditches had a rectangular cross section and were free of any vegetation, whereas natural ditches were vegetated, regardless of the steepness of their banks.

Calling location within a rice-paddy complex: observational study.

To determine niche segregation between the two treefrog species within a rice-paddy complex, we sampled four rice-paddy complexes in Paju. The number of rice paddies in a complex ranged from 59 to 284, with areas between 0.21 and 1.41 km^2. Each complex was investigated for 60 min at a randomly selected time between 16:00 and 05:00 the next day, for a minimum of eight times between 30 May and 1 July 2013. Calling activity was assessed independently for both species for each rice paddy in a complex. Each time a call was heard, the rice paddy from which the

call originated was noted on the map. If several individuals were recorded calling throughout the study period, only one mark was noted on the map.

We measured the following physical characteristics of all rice paddies in the complexes: distance to bush, distance to forest, and distance to road. Distance to bush was defined as the distance from the centre of each rice paddy to the closest vegetation between 30 and 60 cm high and up to 50 cm wide and long. Smaller bushes were excluded from the analysis as they were not constant throughout the breeding season because of agricultural practices. Distance to forest was defined as the distance from the centre of each rice paddy to the closest vegetation with a minimum height of 60 cm and a minimum width of 50 cm, here defined as forest. Due to the remoteness of forest for complex 1, which was farther than the average dispersal distance for treefrogs (Smith and Green 2005), we arbitrarily assigned the distance to forest as 5000 m for complex 1. Distance to road was the distance from the centre of each rice paddy to the closest one-lane concrete or dirt road crossing the rice-paddy complex.

Removal experiment.

The removal experiment was conducted in 2015 because permits are only available every second year at any given site. We used 20 rice-paddy complexes for this study. The sequence in which the two rice paddies were used was randomized. Distance to bank was defined as the distance between the frog and the water line of the closest bank. Distance

to bank for all frogs of the focal species was measured with a range finder (SD 60; Sincon; Taichung, China), at 1 cm resolution. If a frog was less than 50 cm from the bank, a 5 cm resolution was used to prevent disturbance. Distance to bank was positive or negative if the individual was inside or outside, respectively, of the flooded area of the rice paddy. We then proceeded to temporarily remove all frogs of the non-focal species (maximum $n = 11$ for *D. japonicus* and $n = 4$ for *D. suweonensis*). Once non-focal males were removed, advertisement calls were usually produced within 25-30 min at the location of removal by previously silent males or by males that moved from nearby paddies. Thus, a 20-min interval was selected before measuring the locations of the focal species following the same protocol. All frogs removed were released at the place of capture after the experiment.

Calling posture.

The body posture exhibited by calling frogs was investigated in 273 males. Because of the presence of satellite males, only calling males were included in the analysis. Eighteen sites were observed in six rice-paddy complexes between 5 May and 14 July 2013. Two types of body position were recorded. The first one, herein defined as "holding," occurred when a frog had the digits of its forelegs curled around a bearing support (rice seedling) and its hind legs extended. In this position, frogs were usually not parallel to the water. "Sitting" was defined as a frog not

using the digits of its forelegs to hold onto a substrate and having its hind legs in a resting position. Thus, when frogs were "sitting," their bodies were always parallel to the substrate.

Statistical analyses.

To assess whether the calling sites of the two treefrog species were segregated within a rice paddy, we employed a Generalized Linear Mixed Model (GLMM) on the observation data from rice paddies, with distance to bank as a response variable. The predictor variables included season, time of day, species, individual, paddy size, road, and ditch. "Species" was either *D. japonicus* or *D. suweonensis*. "Individual" referred to each individual frog, each one of which was assigned a random number, creating a nominal variable. "Paddy size," "season," and "time of day" were continuous variables. "Season" was the number of days since 16 April 2013, when advertisement calls for both species were heard for the first time that year. "Time of day" was the time after sunset at the closest weather post of the National Weather Service Stations of Korea on the day of monitoring. "Paddy size" was the surface area of a rice paddy, and "paddy width" was the width at which the distance-to-bank measurements were taken. "Road" denoted the absence (0) or presence (1) of a one-lane road, and "ditch" was divided into no ditch (0), natural ditch (1), or concrete ditch (2). Because distance to bank was not normally distributed (Kolmogorov-Smirnov test; $D = 83.00$, $df = 138$, $P < 0.001$),

we additionally used the non-parametric Mann-Whitney *U* test to see whether *D. japonicus* and *D. suweonensis* differed in their calling site locations. None of the variables were correlated with each other ($r \leq 0.16$, $P \geq 0.059$).

A binomial test was used to test the effects of road and vegetation on treefrog locations within a rice paddy. A null hypothesis of equal probabilities for the two categories was assumed for each binomial test. A Chi-square test was used to understand the effect of ditch, with an assumption of equal probabilities among the three categories.

To test the hypothesis of niche segregation at the level of a rice-paddy complex, we tabulated a 2×2 contingency table for each complex. Each rice paddy in a complex had one of four outcomes: both species present, both species absent, only *D. japonicus* calling, and only *D. suweonensis* calling. We employed a *G* test of independence to determine niche segregation between the treefrog species in terms of calling location within a rice-paddy complex. The expected frequencies were calculated in a contingency table, assuming that calling males of the two treefrog species were independent of each other. To determine whether to pool data from the four complexes, we estimated the Mantel-Haenszel common odds ratio. We conducted multinomial logistic regression to determine the factors important for the presence of the two treefrog species. The response variable of the multinomial logistic regression was species

presence (0: none; 1: *D. japonicus*; 2: *D. suweonensis*; 3: both), and the predictor variables were distance to bush, distance to forest, and distance to road. Because the sample size for *D. suweonensis* was small, multinomial logistic regressions were conducted for *D. japonicus* alone and for both species, but not for *D. suweonensis* alone.

In the removal experiment, an individual treefrog was assigned one of three categories for movement: "no movement," "movement towards the centre of the rice paddy," or "movement away from the centre of the rice paddy." We counted the number of individuals in the three categories for each *Hylid* species. We employed a likelihood ratio test of independence to test whether the two treefrog species differed in the directionality of movement in the removal experiment. The expected frequencies of movement were based on the assumption that the two treefrog species did not differ in the directionality of movement. We then tested the difference in the distance moved pre- and post-removal of the non-focal species through ordinary least square regression analysis. The response variable was distance moved, and the predictor variables were focal species, site, frog ID, season, time after sunset, rice paddy width, and number of individuals removed. The reference day for the variable season was 5 May 2015. "Distance moved" was defined as the distance travelled by an individual before and after removal, and could be positive or negative depending on the position of the frog. "Number of individuals

removed" was the number of individuals temporarily removed from the rice paddy during the experiment.

The factors included in the binary logistic regression analysis, conducted in order to determine the factors affecting calling posture during the production of advertisement calls, were: species, snout-vent length (SVL, measured with digital callipers 317-249; Mitutoyo Corp.; Kawasaki, Japan), site, and season. All statistical analyses were computed using SPSS v21.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

Observations within a rice paddy.

To understand niche segregation, we documented the locations of calling individuals of the two treefrog species through acoustic monitoring. When males of both *Dryophytes suweonensis* and *D. japonicus* produced advertisement calls in the same rice paddy during the breeding season, they tended to be segregated from each other. At all locations, calling *Dryophytes japonicus* (n = 123) were located on average 1.4 ± 2.0 m (mean ± SD) from the bank, while calling *D. suweonensis* (n = 15) were on average 12.1 ± 6.9 m from the bank (Fig. 6.2). The average difference between *D. japonicus* and *D. suweonensis* calling sites within a rice paddy was 8.1 ± 3.9 m (min = 3; max = 14). The results of the Generalised Linear Mixed Model indicated that there was a significant

difference between the two species regarding the distance to the bank, but not for individual, season, time of day, paddy size, or presence of road or ditch. In a non-parametric analysis, *D. japonicus* and *D. suweonensis* significantly differed in the location of calling sites within rice paddies (Mann-Whitney *U* test; $U = 83.00$; $n = 138$; $P < 0.001$).

The results of the statistical analysis of the distributions of the two species at the spatial scale of rice-paddy complexes showed that the two species were not significantly different in the likelihood of calling near a ditch ($\chi 2 = 3.86$, $df = 2$, $P = 0.145$) or a road ($\chi 2 = 1.29$, $df = 1$, $P = 0.257$). Thus, the preference for landscape features was analysed together for both species.

Individuals of the two treefrog species ($n = 138$) tended to be present in rice paddies with natural ditches (52.9 %) or without ditches (36.2 %), and tended to avoid rice paddies bordered by concrete ditches (10.9 %). Individuals of the two species were more likely to be found in rice paddies that were not adjacent to roads (74.6 %), rather than in rice paddies adjacent to roads (25.4 %).

Observation within a rice-paddy complex.

The purpose of this analysis was to assess the effect of landscape variables on the distribution of the two species. If niche segregation occurred at the level of a rice-paddy complex, only individuals of one

treefrog species would be found in a given rice paddy. However, the frequencies of paddies with both or neither *Hylid* species present were consistently higher than expected in all four rice-paddy complexes, and the frequency of either species being present alone was consistently lower than expected. The distribution of the two treefrog species differed significantly among the four rice-paddy complexes (Mantel-Haenszel common odds ratio: *ln*(estimate) = 3.197, standard error of *ln*(estimate) = 0.469, $P < 0.001$).

Thus, we tested the hypothesis of niche segregation between the two treefrog species separately for each rice-paddy complex (Table 2.1). The presence of *D. suweonensis* was not independent of that of *D. japonicus* in any of the four rice-paddy complexes (*G* test, likelihood ratio ≥ 8.630, $df = 1$, $P \leq 0.003$). Therefore, frogs of both species were found to be using the same landscape elements at the rice-paddy complex scale. Thus, both treefrog species tend to prefer the same rice paddies and avoid others.

Next, we examined factors important for the presence of the two treefrog species within a rice-paddy complex. Because the number of rice paddies with *D. suweonensis* only was very small, these analyses were conducted for rice paddies with *D. japonicus* only or for those with both species. The results of multinomial logistic regressions suggested that forest was a significant factor for the presence of *D. japonicus*, and

for that of both species. This is explained by treefrogs being more abundant in rice paddies close to forests in complexes 2 and 4 (Table 2.2).

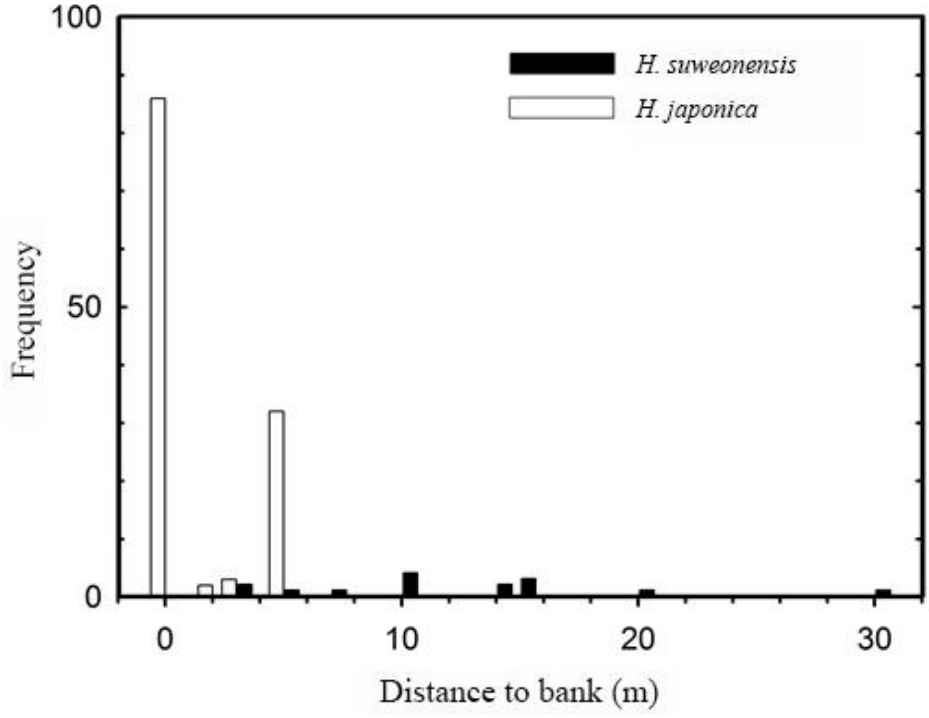


Figure 6.2. Distributions of distance to bank for *D. suweonensis* (solid, n = 15) and *D. japonicus* (open, n = 123) in rice paddies. At night, when male treefrogs produced advertisement calls, they distributed themselves on the rice paddy banks or inside rice paddies. "Distance to bank" was the distance between the bank and the location of a calling treefrog. When a treefrog called on the bank, distance to bank was zero.

In these complexes, rice paddies were located closer to forests than to bushes. Paddies occupied by one or both species were on average 116.6 ± 86.1 m from forests and 139.6 ± 114.2 m from bushes in complex 2 and 61.6 ± 53.6 m from forests and 165.8 ± 127.1 m from bushes in complex 4. In complex 5, however, occupied paddies were, on average, further away from forests (distance to forest: 190.8 ± 159.7 m) than from bushes (distance to bushes: 70.4 ± 64.2 m), and distance to bushes was significantly associated with the presence of *D. japonicus* and of both species. Distance to forests and distance to bushes differed significantly (t = 4.01, df = 516, $P < 0.001$). Roads tended to be significant for the locations of *D. japonicus* and of both species in complexes 4 and 5, and treefrogs tended to call further away from roads in these complexes. Overall, calling location within a rice-paddy complex did not seem to follow a consistent pattern relative to landscape factors.

Removal experiment.

In each rice-paddy complex, two rice paddies with calling treefrogs of both species were randomly chosen. *D. japonicus* was removed from one and *D. suweonensis* was removed from the other. We measured the distance to the closest bank for each individual, pre- and post-removal, to assess the impact of each species on the other one. When calling individuals of the opposite species were removed, focal individuals tended to move towards the position where the opposite species used to

be. When *D. suweonensis* males were removed, 18.1 % of calling *D. japonicus* males moved towards the centre of rice paddies, while 15.7% moved towards the bank and 66.3% did not display any movement (Fig. 6.3b). When calling *D. japonicus* males were removed, 11.4 % of calling *D. suweonensis* males moved towards the centre of rice paddies, whereas 68.2 % moved towards the bank and 20.5 % did not display any movement. The directionality of the movement post-removal was statistically analysed through a likelihood ratio test. The two treefrog species differed significantly in the directionality of movement in the removal experiment (likelihood ratio test: $\chi^2 = 36.71$, $df = 2$, $P < 0.001$; Fig. 6.3a). The two treefrog species moved in the expected directions in the removal experiment, and the distance moved differed significantly between the two treefrog species (excluding individuals not displaying any displacement: t test; $t = 8.54$, $df = 62$, $P < 0.001$; including all individuals: $t = -6.42$, $df = 125$, $P < 0.001$). Calling *D. japonicus* males barely moved in either direction in the removal experiment (0.35 ± 0.39 m, mean ± SD; range: 0.06 - 0.87 m; Fig. 6.3a). However, calling *D. suweonensis* males moved an average of 1.34 ± 1.24 m (mean ± SD, range: 0.08 - 4.76 m) towards the bank. The results of the regression analysis of movement before and after removal showed that only two factors, focal species and time after sunset, were significant.

In summary, the calling locations of the two treefrog species were

segregated within a rice paddy. Calling *D. japonicus* were found on or close to banks, whereas male *D. suweonensis* called from the centre of rice paddies. Male *D. suweonensis* tended to be affected by the presence of *D. japonicus*, but not the other way around.

Calling posture.

Binary logistic regression analysis was used to determine the factors affecting the calling posture during the production of advertisement calls. The factors analysed were species, snout-vent length (SVL), site, and season. Of 240 *D. japonicus* individuals, 239 were "sitting" when producing advertisement calls, whereas 28 of 29 male *D. suweonensis* were "holding." The results of the binary logistic regression showed that the only variable that explained significant variation in calling posture was species, while season, site, and SVL were not significant. That is, the calling posture was significantly different between the two treefrog species. Anecdotally, male *D. suweonensis* produce advertisement calls while sitting on mud above water level in a rice paddy during the period between flooding and rice planting. However, we rarely observed male *D. japonicus* holding onto plants while calling, even if they were sitting on the plants instead of the ground.

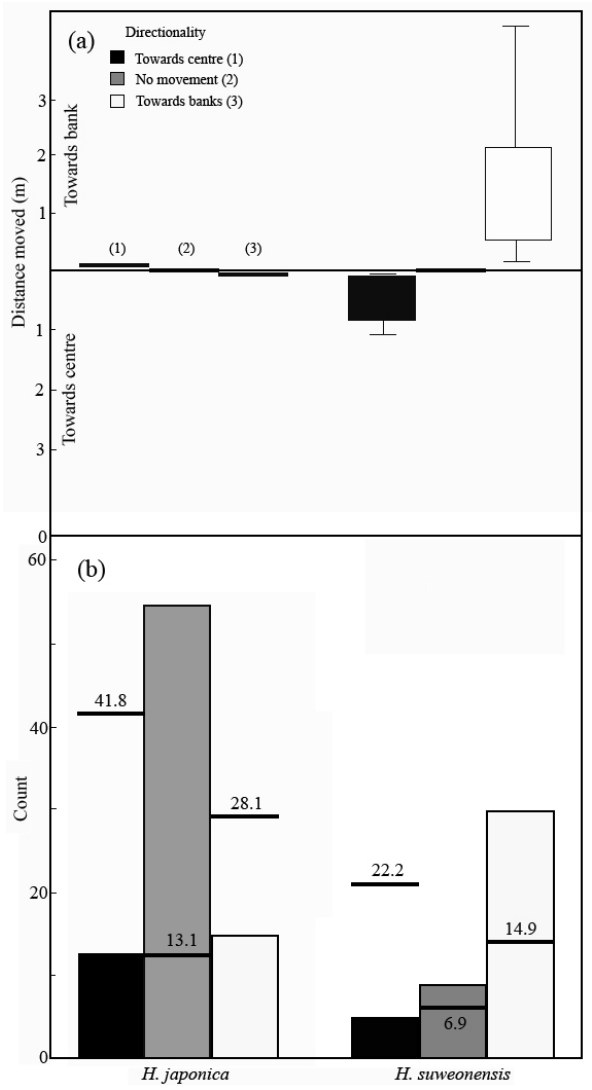


Figure 6.3. (a) Boxplots of the distance moved for the two treefrog species in the removal experiment. Calling locations of the focal species (either *D. japonicus* or *D. suweonensis*) were noted in rice paddies before and after individuals of the non-focal species were removed. Direction of movement was measured as "towards the bank," "no movement," or "towards the centre of rice paddies". (b) Observed counts (bars) of movement in the two treefrog species. Horizontal lines represent the expected counts based on the assumption that the two species did not differ in the direction of movement.

DISCUSSION

The results of our observational studies clearly demonstrate niche segregation between *Dryophytes japonicus* and *D. suweonensis* over calling sites within individual rice paddies. The spatial separation between calling males of the two treefrog species within a rice paddy might be of competitive nature. The calling locations of *D. suweonensis* males were affected by the removal of calling *D. japonicus*, but not the other way around. This result of the removal experiment supports interspecific competition over calling sites in the two treefrog species.

Furthermore, the alternative hypothesis, that the differential use of non-calling site resources in rice paddies between the two treefrog species may result in niche segregation in calling sites is unlikely, as this hypothesis would predict no displacement of calling sites in the removal experiment. Accordingly, *D. suweonensis* males may predate on mayflies that occur mostly inside the rice paddies, whereas *D. japonicus* males feed on non-flying arthropods at the banks of rice paddies. If this hypothesis is true, male movements of either species in and around rice paddies should be correlated with prey density, not with the absence of advertisement calls made by interspecific competitors.

It is unclear what fitness disadvantage may occur when calling male *D. suweonensis* approach the bank of a rice paddy where male *D. japonicus* produce advertisement calls. Male *D. japonicus* may be

physically aggressive towards the calling *D. suweonensis* males. Whether the treefrog species directly compete with each other remains unclear. However, contact between individuals of these two species is rarely observed. Alternatively, the advertisement calls of *D. japonicus* may interfere with those of *D. suweonensis* in close proximity. Although the fitness consequences of competition have not yet been determined, niche segregation and adjustment of calling sites in the absence of competitors are consistent with the predictions of interspecific competition between the two species.

Competition over calling sites between calling males of the two treefrog species was asymmetric. Male *D. japonicus* are 12.9 % larger than *D. suweonensis* (Park et al. 2013), and *D. japonicus* is more abundant than *D. suweonensis* throughout the known range of *D. suweonensis* (Roh et al. 2014). The physical superiority and abundance of *D. japonicus* may provide a competitive advantage over the sympatric species, allowing *D. japonicus* to dominate the banks of rice paddies for call production. The effectiveness of a male's advertisement calls in attracting a female may be severely reduced by the loud background noise produced by a large group of *D. japonicus* males. This may cause *D. suweonensis* to move away, towards a less ideal habitat, and consequently lead to reduced mating opportunities or interspecific mating. More studies are needed to strengthen the hypothesis of asymmetric competition between the two

treefrog species over calling sites, including playback experiments in which simulated conspecific calls affect the calling locations of the two treefrog species.

The spatial arrangement of calling sites in a rice paddy resulted in *D. japonicus* males calling at the edges of rice paddies, along a line parallel to the water line, because of territorial boundaries. This creates a "barricade" that females of the central species, i.e. *D. suweonensis*, have to cross before reaching conspecific males. This arrangement may decrease the number of females from the central species reaching the breeding area without interference from males of the edge species. This spatial arrangement is also seen for *H. gratiosa* and *H. cinerea*, two North American *Hylids*, where males of the former species call from the pond proper, and males of the latter species call from outside the water (Lamb 1987).

Furthermore, because of the possibility of hybridisation (Kuramoto 1984) and erroneous mate selection by females (Brattstrom and Warren 1955), the spatial arrangement presents a challenge to *D. suweonensis* species integrity. The predominance of the *D. japonicus* population may facilitate directional introgression during hybridisation (Lamb and Avise 1986). In this case, if hybridisation occurs in the wild, male *D. japonicus* mating with female *D. suweonensis* may predominate over the reciprocal combination. Such a directional bias may lead to

backcrossing in later generations, thus leading to cytonuclear disequilibrium and creating introgression patterns comparable to those found in *Bufo viridis* (Colliard et al. 2010).

We did not detect any evidence of niche segregation with respect to time of day or season. Males of both species typically produce advertisement calls at the same time. Anecdotal observations (personal observations by A. Borzée and Y. Jang) suggest that *D. suweonensis* advertisement calls are almost always accompanied by *D. japonicus* advertisement calls in the same rice paddy. Because this study was limited to a maximum of 32 consecutive days, there is a possibility of niche segregation in breeding seasons, outside of the study period. However, a more extensive, nine-week study showed that the calling seasons of both treefrog species completely overlap (Roh et al. 2014). Thus, temporal separation is unlikely to be the mechanism isolating *D. japonicus* and *D. suweonensis* populations.

Segregation of calling locations within a rice paddy may directly affect body posture during the production of advertisement calls. Male *D. suweonensis* call from the centre of rice paddies, where they cannot sit on a hard substrate and must hold onto rice seedlings. Conversely, male *D. japonicus* mainly call from rice paddy banks, where they can sit on a substrate during call production. In the only semi-natural habitat known for *D. suweonensis* (Borzée and Jang 2015), which is characterised by a

network of marshes, grasses, and trees, male *D. suweonensis* assume a sitting posture during the production of advertisement calls (Borzée et al. 2016b). The holding posture is exhibited in rice paddies, where *D. suweonensis* are forced into the centre of the water body because of competition from *D. japonicus*. We believe that the sitting posture is preferred for *D. suweonensis* in the heterogeneous semi-natural habitat where *D. suweonensis* can avoid direct contact with *D. japonicus*. If interspecific competition is driving the arrangement of calling locations in the two treefrog species in rice paddies, holding may be an adaptation for calling at the centre of rice paddies in *D. suweonensis*.

This study suffers from a lack of intraspecific controls in the removal experiment, a low *D. suweonensis* sample size, and a limited number of sampling areas. The effect of removal of intraspecific calling males should be compared to the effect of removal of interspecific calling males in a future removal experiment. Observational and removal studies should be replicated over areas that represent the whole range of *D. suweonensis*. Another potential problem of this study was that the spatial correlations of rice paddies within a rice-paddy complex may bias the effects of landscape features on the positioning of the two treefrog species.

Neither treefrog species was randomly distributed with respect to landscape features within a rice paddy. Within rice-paddy complexes, rice

paddies with only *D. japonicus* and with both species generally had similar landscape features. These findings suggest similar ecological requirements for both treefrog species in a rice-paddy complex. The only landscape features correlated with the presence of the two species were related to vegetation. Forest and bushes are hypothetical hibernation sites (Pellet et al. 2006), and breeding sites need to be within an average of 2000 m of these features (Smith and Green 2005; Angelone and Holderegger 2009) for the annual migration between hibernating and breeding sites. The distance from rice paddies to the closest vegetation, defined as a combination of the forest and bush variables, was similar for both species. This result can be explained by a common need for shelter, resting places, and predator avoidance (Fujioka and Lane 1997).

The finding that both treefrog species tended to prefer the sides of rice paddies with no ditch or a natural ditch to those with a concrete ditch may be important for conservation of anuran species, including the endangered *D. suweonensis*. A tracking experiment showed that both treefrog species spent their days on rice paddy banks resting, hiding, and feeding (Borzée et al. 2016a). Unlike natural ditches, concrete ditches typically remain dry, which limits grass growth. This finding differs from that of a study in Japan (Naito et al. 2012), in which ditches did not have an effect on the temporal or spatial distribution of anuran species, including *D. japonicus*. However, this study examined the presence of

treefrogs in rice paddies, without examining which side of the rice paddies attracted the treefrogs. Typically, only one side of a rice paddy is bordered by a concrete ditch, so frogs can remain close to the sides of rice paddies without concrete ditches.

The information obtained in this study is critical for maintaining species diversity and conserving endangered *D. suweonensis*. Traditionally, differences in male advertisement calls and female selectivity are regarded as the driving forces for both speciation and maintenance of species diversity in anurans. However, asymmetric competition can influence population dynamics and community structure by affecting the population size of rare or endangered species (Parris and Semlitsch 1998). In rice paddies, *D. suweonensis* occurs with other frog species, including *D. japonicus*, the black-spotted pond frog *Pelophylax nigromaculatus*, and the Seoul golden frog *Pelophylax chosenicus*. Several frog species may compete with each other for calling locations, and dynamic interactions among species may determine breeding locations in a multi-species anuran community.

Chapter seven

RELATIONSHIP BETWEEN AGRO-ENVIRONMENTAL VARIABLES AND RICE PADDY USE BY HYLIDS.

ABSTRACT

When natural wetlands are destroyed, many anuran species are forced to breed together in alternative habitats such as rice paddies. Due to different origins, the environmental pressure on specific anuran species may largely depends on the degree of similarity between their original natural habitat and rice paddy. In the Republic of Korea, most natural wetlands have been replaced by rice paddies, which became substitute breeding sites for Hylids. We conducted field surveys from the beginning of the breeding seasons, and for two weeks after its peak, for the endangered *Dryophytes suweonensis* and the numerous *D. japonicus*. We recorded presence, number of individuals and calling indices for the species. The purpose of this study was to describe the impact of agricultural practices and environmental variables on the initiation of the breeding period for the two Korean Hylid species. We hypothesized *D. japonicus* to start breeding earlier than *D. suweonensis*, due to a broader behavioural spectrum resulting from relatively heterogeneous habitats, while *D. suweonensis* would have been originally been breeding solely in floodplains. The results of our analyses demonstrated that the rice

cultivation phase was the most important factor to predict the calling activities of both species. Furthermore, the peak calling activities of both treefrog species matched with the optimal hydroperiod in the rice paddy, although *D. japonicus* started breeding earlier than *D. suweonensis*. Besides, *D. japonicus* breeding behaviour was influenced by environmental variables such as temperature, whereas *D. suweonensis* seems to require the plantation of rice seedlings to initiate breeding activities. Indeed, breeding activity of *D. suweonensis* seemed to be triggered by flooding of rice paddies for agricultural purposes. It is therefore important to provide adequate breeding sites to this endangered species early enough in the season, as no natural habitat is left. Therefore, as both *Dryophytes* species see their breeding activities influenced by agro-environmental variables, this study highlights the importance of also preserving anthropogenically modified landscapes for the conservation of species.

INTRODUCTION

Most amphibians species are found in wetlands, typically situated in low-lying plains (Dodd Jr 1992; Semlitsch 1998; Quesnelle et al. 2015). Unfortunately, low-lying plains, along with their wetlands, are the most adequate environment for large-scale rice cultivation. Worldwide, the primary reason for wetland loss is the conversion to agricultural fields

(Czech and Parsons 2002; Juliano 1993). For instance, 50 % of wetlands were converted to agricultural fields in North America and Europe (Millennium Ecosystem Assessment 2005), and 90 % of wetlands were converted in Brazil (Machado and Maltchik 2010). In 2003, Asia accounted for about 89 % of rice paddies in the world (FAO Stat 2004). In the Republic of Korea, out of the 22% of landmass available for crop production, 90% of the arable lands are used for rice production (US Library of Congress 2015). Wetlands that are not used for agriculture are often targeted for urbanisation, as lowlands run by fresh water are attractive to both humans and other species (Huston 1993; Mitsch and Gosselink 2007).

Amphibians are opportunistic breeders (Husté et al. 2006; Colding et al. 2009; Hamer et al. 2012; Scheffers and Paszkowski 2013; Holzer 2014b), and extensive agricultural land expansion has caused wetland breeders to become rice paddy breeders. Rice paddies present advantages for breeding amphibian, such as a prolonged yearly hydroperiod. In fact, many amphibian species are solely or heavily relying on rice paddies for breeding (Hobbs et al. 2009; Machado and Maltchik 2010; Fujioka and Lane 1997; Magle et al. 2012; Naito et al. 2013; Holzer 2014a). Nevertheless, the conservation of natural wetlands is a necessity as some species are restricted to natural wetlands (Dodd and Cade 1998; Silvano and Segalla 2005), or do not fare as well in rice paddies as in

swamps (Hasegawa et al. 2000; Osawa and Katsuno 2002). The decrease in natural wetlands highlights the need for surrogate habitats for wetland-based amphibians (Rosenzweig 2003; Hobbs et al. 2011). Besides, rice paddies can be used as a connecting matrix between populations breeding at natural sites (Machado and Maltchik 2010), and therefore limiting the effects of fragmentation derived from human development (Ricketts 2001; Semlitsch and Bodie 2003; Kupfer et al. 2006).

Some agricultural areas for rice cultivation boast relatively high diversity of species (Burhanuddin 1992; Brouder and Hill 1995; Davis 1994; Elphick and Oring 1998; Czech and Parsons 2002; Getzner 2002; Holzer 2014a), such as shown for wading birds in the Mediterranean basin (Fasola and Ruiz 1996), or for amphibians in Brazil (Machado and Maltchik 2010). The variation in rice paddy management can have a different impact on amphibian populations across regions. For instance, flooding of rice paddies during the fallow phase in Brazil was not associated with amphibian richness (Machado and Maltchik 2010), while it may be important in California (Brouder and Hill 1995;Elphick and Oring 2003). Asia, with the largest rice plantations in the world, follows this positive trend of association between rice cultivation and anuran biomass (Bambaradeniya and Amerasinghe 2003), supported by examples for amphibians in Japan and Vietnam (Naito et al. 2013; Holzer 2014b). However, a study showed that modern rice farming practices had a

negative impact on species such as *Rana japonica* and *R. porosa* in Japan (Fujioka and Lane 1997).

Anuran assemblages are strongly influenced by abiotic and biotic factors (Pechmann et al. 1989; Rome et al. 1992; Duellman and Trueb 1994; Babbitt and Tanner 2000; Bertoluci and Rodrigues 2002). Here, we try to understand whether and how the breeding biology of Korean treefrogs is limited by farming practices including water availability at rice paddies. Land use conversion to rice paddies since the development of human agriculture (such as described by Fuller et al. 2007; Fuller et al. 2008) might have affected the functional equilibrium between the endangered *Dryophytes suweonensis* and the numerous *D. japonicus* (Borzée et al. 2015b), a genus previously attributed to *Hyla* (Duellman et al. 2016). It is highly likely that *D. japonicus* relies on forested habitats during the non-breeding season (Borzée et al. *in review-a*), while *D. suweonensis* might be restricted to wetlands for both breeding and non-breeding seasons (Borzée and Jang 2016a). Thus, prior to rice farming, lowlands would have been providing most of the habitats for *D. suweonensis,* which requires shallow and lentic water bodies (Borzée and Jang 2015). This new alternative breeding ground was also colonized by *D. japonicus*, relying on its already wider acceptance of habitats (Maeda and Matsui 1999; Naito et al. 2013), and resulting in increases in population sizes (Borzée et al. 2015b). Thus, the two treefrog species were

forced into breeding together in rice paddies, despite their segregated evolutionary history, such as seen by the different response to predators (Kim 2016). We hypothesized that *Dryophytes japonicus* would start breeding earlier than *D. suweonensis*, as the species originates from habitats with reduced hydroperiod and is evolutionarily advantaged by timely exploiting all water resources available. Thus, the secondary purpose of this study was to assess the viability of rice paddies and agricultural practices for the survival of the endangered *D. suweonensis* and the numerous *D. japonicus*.

MATERIAL AND METHODS

The beginning of the study preceded the breeding seasons of both *Dryophytes japonicus* and *D. suweonensis* (Naito et al. 2013; Roh et al. 2014). The setting of modern rice paddies leads to a specific grouping of rice paddies, here referred to as rice-paddy complex, with a central lane that follows the central ditch running mostly straight through the complex for irrigation purposes. This lane usually is along the longest and straightest line available, as following the centre of the valley used for the rice-paddy complex. Rice paddies are delimited by levees roughly 40 cm wide and 20 to 60 cm high, covered with grasses used for basking, foraging, and sheltering by amphibians (Fujioka and Lane 1997, Borzée et al. 2016a). In the Republic of Korea, *Dryophytes spp.* and *Pelophylax spp*.

are typically found together on levees during their breeding seasons (Borzée et al. 2016a).

Ten rice-paddy complexes where *D. suweonensis* was known to occur were selected as study sites (Roh et al. 2014; Borzée et al. 2016c; Borzée et al. 2017b). However, *D. suweonensis* was not heard calling at two of the selected complexes (Fig. 7.1). Because the advertisement calls of *D. japonicus* and *D. suweonensis* are species-specific (Park et al. 2013), we employed acoustic monitoring to assess the population sizes for the two species (Dorcas et al. 2009; Weir et al. 2005; Pellet et al. 2007; Petitot et al. 2014; Moreira et al. 2016). Surveys were conducted twice a week over a 60-day study period, starting on 16 April 2014 in the city of Paju, Gyeonggi Province, Republic of Korea (Fig. 7.1), and resulting in 15 surveys per site during the study period. Here, a site was defined as a locality where surveys were conducted for four survey points, as well as a walking survey at the scale of the rice-paddy complex. Two sampling techniques were used for acoustic monitoring: transect and point surveys. Each of the 10 sites was surveyed through one transect and four independent point surveys, one at each of four individual rice paddies. Each point survey was at least 500 m away from the nearest point to insure independence of data, as an individual *D. suweonensis* can be heard up to 250 ± 45 m away (Borzée et al. 2017b).

For both point and transect surveys, we documented the phases of

rice cultivation: fallow, ploughed, agricultural flood, tilled, and rice plantation. Two other variables were annotated separately, due to their non-cyclical and random occurrence: pre-seedling production and rainwater partial flooding.

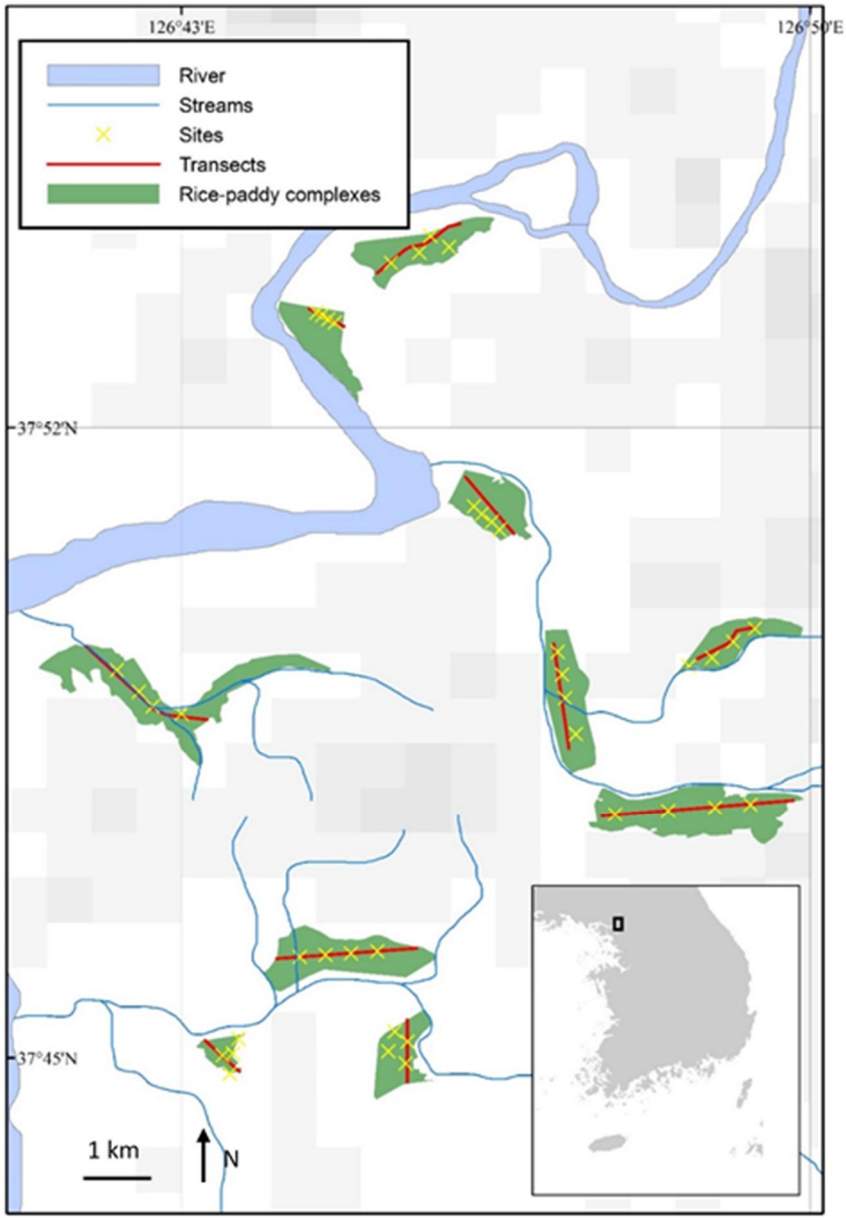


Figure 7.1. Map representative of the 40 sites and 10 complexes where data collection took place over a 60-day study period, starting on 16 April 2014. All sites are within the administrative area of Paju, Gyeonggi Province, Republic of Korea.

Pre-seedling production refers to the period when all seedlings are grown together in restricted areas, under tarpaulins for protection against the cold, and usually in a single low lying rice paddy for ease of irrigation. These agricultural processes were deemed important for the breeding of the two *Dryophytes spp.* due to the disturbance by agricultural equipment such as tractors, the hydroperiod (Babbitt and Tanner 2000), and diversity and abundance of juvenile amphibians (Pechmann et al. 1989). Finally, plantation of rice seedling may be beneficial for male *D. suweonensis*, as they use them as perches from which to call (Borzée et al. 2016b). At the beginning of each survey, we also recorded date, time of day, air temperature (°C) and relative humidity (%) due to their importance to call production (Rome et al. 1992). Data for two rice-paddy complexes could not be recorded for the ninth survey, and are treated as missing data for subsequent analyses.

Point surveys

Aural point surveys were conducted at each of the 40 individual rice paddies. After arrival at a survey point, 5 min were spent waiting quietly and the next 5 min were used to record the calling activity of hylids from the survey point. All calling individuals heard were recorded, although potentially outside of the focal rice paddy. In previous studies, 5 min were shown to be sufficient to monitor the calling activities of many species (Gooch et al. 2006; Weir et al. 2005; Petitot et al. 2014). The

calling activity was recorded in two ways: first we counted the number of calling individuals within the rice paddy selected. This procedure was repeated a minimum of 12 times to ensure that all calling individuals were accounted for. This method was deemed adequate for *D. suweonensis* as the species is not abundant, *i.e.* the maximum number of individuals was 10 (mean = 0.21, SD = 0.72) individuals per survey point, and advertisement calls are clear and unique. For *D. japonicus*, however, calling males regularly exceeded 10 individuals, and this method was not adequate to count calling individuals. Consequently, the maximum number of individuals counted was set at 10, based on the maximum number of *D. suweonensis* individuals recorded. Instead, we relied on the calling index (CI) such as defined by Mossman et al. (1998) and modified by Roh et al. (2014) . The number of calling individuals is classified in four categories: 0: no individuals are calling; 1: calls are not overlapping and it is possible to count the number of individuals calling; 2: calls are overlapping but it is still possible to count the number of individuals calling; 3: it becomes impossible to count the number of individuals. This method was appropriate for the numerous *D. japonicus*, but not optimal for the low number of calling *D. suweonensis* as describing too little variation. The results of both methods were analysed jointly and separately.

Transect surveys

Following the point survey of all four points at a rice-paddy complex, we moved to one end of the central lane of the same rice-paddy complex. The line transect surveys were conducted at a maximum speed of 80 m/min for a minimum of 10 min (~ 5 km/h, brisk walking) along the central lane. The presence or absence of individuals was binary encoded for each species. The line transects were not centrally located in complex 6 and 10 (Fig. 7.1), but they were still within hearing ranges of the entire rice-paddy complexes. We empirically tested for aural detection of the two species (n = 20) and established detectability at 250 m (± 45).

Statistical analysis

The first set of analyses determined the relationship between agro-environmental variables and the calling activity of the two species at two scales: survey point and rice-paddy complex. To do so, we numerically encoded "cultivation phase" into an ordinal variable ranging from 1 to 5, respectively matching with fallow phase, ploughed, agricultural flood, tilled and rice plantation. Additionally, we binary encoded pre-seedling production and rain water partial flooding. To test whether environmental and agricultural variables were important for the production of advertisement calls for both species, we tested for the occurrence and the calling activity in number of individuals and CI. Both regression and General Linear Models (GLM) were used due to the

different types of data, with the same dependent variables.

(Binomial regressions) The occurrences of *D. japonicus* and *D. suweonensis* were analysed using binary logistic regressions at two spatial scales: survey points and line transects. The occurrence of the two species was binary encoded at both scales. Thus, there were four separate binary logistic regression. Besides, all independent variables were on either continuous, ordinal or nominal scales: temperature, humidity, cultivation phase, pre-seedlings, rain flood and survey points in interaction with rice-paddy complexes, and rice-paddy complex. All variables measured were spatially or geographically independent of each other. We did not include season and time of day in this analyses as the surveys had been designed to increase detection based on temporal and seasonal detectability.

(Generalised Linear Model) When determining the statistical test for the calling activities of *D. suweonensis*, here assessed through the number of calling individuals, we first determined the normality of the data with the Kolmogorov-Smirnov test with Lilliefors Significance Correction (D_{592} = 491, $p < 0.001$), but the normal distribution of residuals through the analysis of QQ plots, and determined the homogeneity of variance with Levene's test for homogeneity of variances ($F_{1,590}$ = 149.40, p = 0.059). We then visually noticed four outliers through the analysis of box-plots for the number of calling *D. suweonensis*. We decided to ignore this violation of assumptions for Linear Models as concerning four out of 592 data

points, and selected a Generalised Linear Model with an ordinal logistic response variable for the number of calling *D. suweonensis*. The Generalised Linear Model was run with factor and covariates set under a main effect model. The predictor variables were temperature, humidity, and cultivation phase as covariates, and pre-seedlings, rain flood and survey points nested within rice-paddy complexes and rice-paddy complexes as factors. All variables measured were spatially or geographically independent of each other; and we did not include season and time of day in this analyses as the surveys had been designed to increase detection based on temporal and seasonal detectability.

(GLM) The calling activity of *D. japonicus*, inferred through the Calling Index was analysed through a General Linear Model (GLM). Independent categorical variables (pre-seedlings, rain flood and survey points nested within rice-paddy complexes and rice-paddy complexes) were set as fixed factors and independent linear variables (temperature, humidity, and cultivation phase) as covariates. When testing for assumptions for the model, we did not notice any outlier through the analysis of box-plots. Besides, we determined the normal distribution of the data through the graphical analysis of residuals on QQ plots, and determined the homogeneity of variance with Levene's test for homogeneity of variances ($F_{(1,590)} = 28.77$, $p = 0.051$). Here as well, all variables measured were spatially or geographically independent of each other, and we did not

include season and time of day in this analyses for the same reasons.

(Repeated measure ANOVA) To be able to conduct the subsequent analysis we analysed the dataset with the purpose to segregate the calling activity in pre-, post-, and lekking periods for further analysis, as the effect of environmental variables changes based on lekking periods (Wells 2010; Höglund and Alatalo 2014; Kim 2015a). We employed a repeated measure ANOVA with survey as the predictor variable to assess the patterns of temporal variations in calling activity of the two species. Because the number of calling *D. suweonensis* was significantly correlated with all individual counts and presence variables ($P < 0.001$, $n = 588$; $R > 0.18$), the number of calling *D. suweonensis* was used as the response variable representative of the lekking activity for both species. We tested for homogeneity of variance with Levene's test and sphericity assumption with Mauchy's test. We ran the repeated measures ANOVA with the Greenhouse-Geisser correction (Scheiner and Gurevitch 2001) due to the violation of the assumption of sphericity. Furthermore, compound symmetry (homogeneity of the variance-covariance matrix) was assumed for this analysis. The analysis was set with 15 levels of within subject variables and the comparison of main effects. The results of the repeated measures ANOVA are presented here for ease of understanding of further analyses. The repeated measure ANOVA for the number of calling *D. suweonensis* for each survey demonstrated that the mean calling activity

differed significantly between surveys ($F = 6.06$; $df = 3.46$; = 137.94; $P < 0.001$). *Post-hoc* analyses on a case by case basis, through the comparison of main effects (Table 7.1), showed that surveys 8 to 13 were grouped together in a non-significantly different group (mean $p = 0.28$), although significantly different from other replicates on the same period (mean $p = 0.05$). This highlights the peak activity of the breeding season, and there was no significant variations in the number of individuals detected through aural surveys for *D. suweonensis*. We therefore qualified the replicates 1 to 7 of *pre-lekking*, the replicates 8 to 13 of *lekking period* and the replicates 14 and 15 of *post-lekking*, following Kim (2015).

Following the division of the lekking period, pre- and post-lekking were analysed through binary logistic regressions for each species using the presence data at the complex as dependent variables. This choice of dependent variable was made due the fact that the CI is in majority 0 and 1 at this time period, the number of individuals for a survey point is too conservative for *D. japonicus* and presence in the complex described a larger variation that the one at the survey point. The variables used for the pre-lekking analysis were season, temperature, humidity and cultivation phase. For the post-lekking analysis, only season, temperature, humidity and agricultural flooding were used as all other variables were constant, *i.e.* agricultural practices such as tilling are required only once per season.

The lekking period was analysed through multinomial logistic

regressions, based on the CI of the species. The CI was set as dependent variable as it was assessed to be the best fitting factor due to the high variability per class for this time period, *i.e.* CI from 0 to 3. The cultivation phase was set as factors, while season, temperature and humidity were set as covariates, under a main effect model.

Besides, we graphically plotted all variables significant from any of the analysis against the calling activity of the two species to obtain a graphical representation of the variables important for the calling activity of the two species. We used the presence/absence of the species at survey points, and not the number or CI, as a single individual calling was assessed to be representative of the breeding status of the species due to the commonalities of the physiological processes involved (Sugimoto and Jiang 2008) and the absence of outlier that would have falsified our analysis.

Finally, we assessed the relationship between the two species to ensure that their calling activities were not influencing each other, and that agricultural practices were the reasons for the patterns described. This was tested as *D. japonicus* is known to significantly influence *D. suweonensis* calling site selection (Borzée et al. 2016b). The dataset was not collected in a way that enables statistical tests to fully address the question, but correlations were tested for consistency. We used Pearson correlations to compare the calling activity of the two species, independently for each of

the 15 replicates. All analyses were conducted in SPSS (IBM SPSS Statistics Inc., Chicago USA).

RESULTS

Environmental conditions changed drastically as the rice cultivation phases progressed. During the study period, the average temperature when no individual was surveyed at the rice-paddy complex scale was 13.88 ± 5.58 °C (mean ± SD) for *D. suweonensis* and 13.70 ± 6.56 °C for *D. japonicus*. The temperature was on average higher for *D. suweonensis* (19.78 ± 4.00 °C) than for *D. japonicus* (16.91 ±5.16 °C) when the two species were present. The pattern was the same for temperature and occurrence at single survey points for both species, but also for humidity at the two scales and for both species. However the temperature and humidity were similar during fallow (11.62 ± 2.33 °C; 73.62 ± 9.54 % rH), ploughed phases (11.50 ± 3.34 °C; 61.51 ± 13.41 % rH) and tilling (11.70 ± 2.74 °C; 61.53 ± 13.54 % rH), while clearly different during flattening (18.20 ± 5.88 °C; 75.43 ± 17.43 % rH) and agricultural flooding (20.53 ± 3.97 °C; 84.61 ± 11.03 % rH). We thus investigated the relationship between environmental and temporal variables on the calling activity and phenology of the two treefrog species.

Table 7.1. Rounded *P*-values for pairwise comparisons of main effects of the repeated ANOVA on the bi-weekly surveys at each of the sites. The framed box represents the replicates falling in a different category in comparison with the others.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1				0.10	0.32		0.32	0.09	0.01	0.01	0.01	0.03	0.02	0.08	0.32
2				0.10	0.32		0.32	0.09	0.01	0.01	0.01	0.01	0.02	0.08	0.32
3				0.10	0.32		0.32	0.09	0.01	0.01	0.01	0.01	0.023	0.08	0.32
4	0.10	0.10	0.10		0.74	0.10	0.08	0.21	0.01	0.01	0.01	0.10	0.24	0.64	0.26
5	0.32	0.32	0.32	0.74		0.32	0.53	0.18	0.01	0.01	0.01	0.08	0.19	0.52	0.53
6				0.10	0.32		0.32	0.09	0.01	0.01	0.01	0.01	0.02	0.08	0.32
7	0.32	0.32	0.32	0.08	0.53	0.32		0.12	0.01	0.01	0.01	0.01	0.04	0.12	0.99
8	0.09	0.09	0.09	0.21	0.18	0.09	0.12		0.58	0.85	0.22	0.51	0.38	0.27	0.11
9	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.58		0.44	0.34	0.02	0.02	0.01	0.01
10	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.85	0.44		0.14	0.01	0.11	0.04	0.01
11	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.22	0.34	0.14		0.01	0.01	0.01	0.01
12	0.01	0.01	0.01	0.10	0.08	0.01	0.10	0.51	0.02	0.01	0.01		0.62	0.18	0.01
13	0.02	0.02	0.02	0.24	0.12	0.02	0.05	0.38	0.01	0.11	0.01	0.62		0.16	0.03
14	0.08	0.08	0.08	0.64	0.52	0.08	0.17	0.27	0.01	0.04	0.01	0.18	0.16		0.13
15	0.32	0.32	0.32	0.26	0.53	0.32	0.99	0.11	0.01	0.01	0.01	0.01	0.03	0.13	

Environmental and agricultural variations

(Binomial regressions) The logistic regressions performed to ascertain the effects of agricultural and environmental factors on the occurrence of either species at the survey points and rice-paddy complexes were statistically significant: occurrence of *D. japonicus* at survey points: $\chi^2_{(7)} = 184.66$, $p < 0.001$; occurrence of *D. suweonensis* at survey points: $\chi^2_{(7)} = 98.87$, $p < 0.001$; occurrence of *D. japonicus* at complexes: $\chi^2_{(7)} = 74.36$, $p < 0.001$; occurrence of *D. suweonensis* at complexes: $\chi^2_{(7)} = 217.04$, $p < 0.001$. The models explained between 16.7 and 42.4 % (Nagelkerke R^2) of the variance in occurrence and correctly classified between 73.0 and 87.5 % of cases. For both species, and at both spatial scales, the cultivation phase was significant (Fig. 7.2, 7.3). Temperature and rain flood were also significant for *D. japonicus* at both site and complex scale (Fig. 7.5), while the only additional significant variable for *D. suweonensis* was temperature at the rice-paddy complexes, and rice paddy-complex at the single survey point scale (Table 7.2). Results for *post-hoc* analyses are presented below the next analysis due to the commonalities in results.

These results demonstrate that for both species, and despite the different scales used, the occurrence at survey points was the variables the least affected by agricultural practices, followed by the occurrence at rice-paddy complexes, and finally by the number of individuals and the CI. When considering *D. japonicus* alone, the variation was minimal for

occurrence at survey points, with three factors significantly important for the number of calling individuals and CI (n = 5). When *D. suweonensis* was considered alone in the analysis, a single environmental variable was significant for occurrence at a survey points, and the maximum number of factors was three for occurrence in rice-paddy complex.

(Generalised Linear Model) The model to assess the effect of variables on the number of calling *D. suweonensis* was a good fit for the data (Omnibus Test; Likelihood ratio $\chi^2_{(44)} = 197.46$, $p < 0.001$). The results of the test (Table 7.3) show that temperature and cultivation phase are significant (Fig. 7.3). Besides, both site nested within complex and rice-paddy complex are significant for the number of calling *D. suweonensis*, but here only highlight the variation in population size between sites.

(GLM) To test if the variable fit into the model showed that all variables were significant, with humidity as the least informative variable: cultivation phase: $\Lambda = 0.77$, $F_{(16,1653)} = 9.34$, $p < 0.001$; pre-seedling $\Lambda = 0.98$, $F_{(4,541)} = 2.46$, $p = 0.044$; rain flood $\Lambda = 0.97$, $F_{(4,541)} = 3.72$, $p = 0.005$; temperature $\Lambda = 0.98$, $F_{(4,541)} = 2.91$, $p = 0.021$; humidity $\Lambda = 0.99$, $F_{(4,541)} = 1.97$, $p = 0.098$; complex(survey point) $\Lambda = 0.59$, $F_{(156,2158)} = 2.46$, $p = 0.044$ and complex $\Lambda = 0.72$, $F_{(4,541)} = 2.84$, $p = 0.048$. The results of the GLM (Table 7.4) show that temperature, cultivation phase, pre-seedlings, site nested within complex and rice-paddy complex are significant (Fig. 7.5).

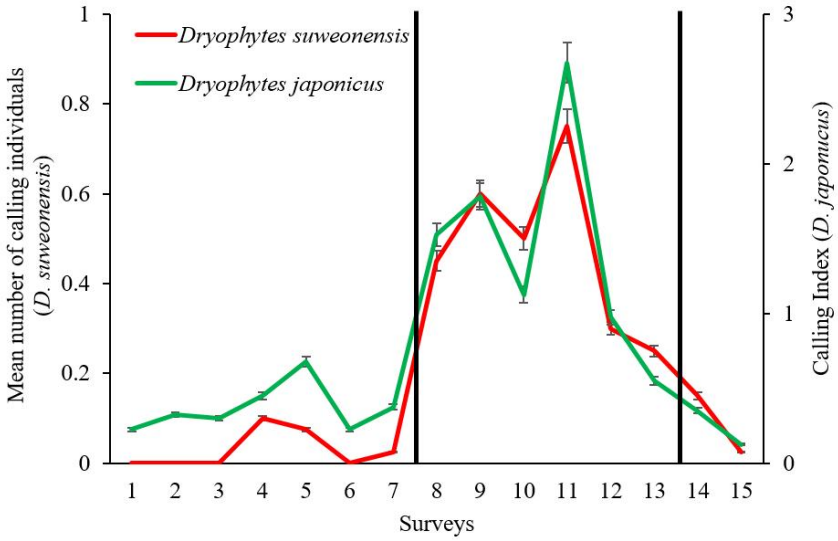


Figure 7.3. Mean number of calling individuals and the mean CI over the study period, covering 60 day, and starting on 16 April 2014. The lekking activity is divided in pre-lekking for surveys 1 to 7, lekking for the surveys 8 to 13 and post lekking for the surveys 14 and 15. The vertical black bars represent the separations between the three lekking periods.

Table 7.2. Results of the Binominal regressions assessing the occurrence of *Dryophytes*

n = 592	B	S.E.	Wald	df	*p*-value
Occurrence *D. japonicus* at single survey points					
Temperature	-0.06	0.03	5.54	1	**0.019**
Humidity	-0.02	0.01	3.73	1	0.053
Cultivation phase	1.51	0.15	106.67	1	**< 0.001**
Pre-seedlings	-0.16	0.35	0.12	1	0.664
Complex x survey point	0.01	0.01	2.31	1	0.069
Complex	-0.22	0.15	2.27	1	0.132
Rain flood	2.42	0.54	19.69	1	**< 0.001**
Occurrence *D. suweonensis* at single survey points					
Temperature	-0.04	0.04	1.08	1	0.298
Humidity	-0.01	0.01	0.06	1	0.938
Cultivation phase	1.57	0.24	43.03	1	**< 0.001**
Pre-seedlings	0.81	0.48	2.77	1	0.096
Complex x survey point	-0.01	0.01	3.45	1	0.063
Complex	0.47	0.22	4.46	1	**0.035**
Rain flood	-17.80	9089.36	0.01	1	0.998
Occurrence *D. japonicus* in complex					
Temperature	0.06	0.02	6.71	1	**0.010**
Humidity	-0.01	0.01	1.22	1	0.255
Cultivation phase	0.43	0.11	15.87	1	**< 0.001**
Pre-seedlings	-0.11	0.32	0.12	1	0.729
Complex x survey point	-0.01	0.01	0.06	1	0.804
Complex	-0.10	0.14	0.55	1	0.458
Rain flood	2.47	1.03	5.71	1	**0.017**
Occurrence *D. suweonensis* in complex					
Temperature	0.13	0.03	22.53	1	**< 0.001**
Humidity	-0.01	0.01	0.31	1	0.578
Cultivation phase	0.82	0.13	37.45	1	**< 0.001**
Pre-seedlings	0.54	0.36	2.26	1	0.133
Complex x survey point	-0.01	0.01	0.36	1	0.546
Complex	0.29	0.16	3.36	1	0.067
Rain flood	0.33	0.61	0.29	1	0.586

suweonensis and *D. japonicus* in relation with environmental and agricultural variables.

Table 7.3. Results of the Generalised Linear Model conducted to assess the significance of environmental and agricultural variables on the number of calling *Dryophytes suweonensis* at sites (n = 592).

	χ^2	df	p-value
Site(Complex)	52.92	30	**0.006**
Complex	43.54	9	**< 0.001**
Pre-seedlings	1.62	1	0.204
Rain flood	1.27	1	0.259
Temperature	4.29	1	0.038
Humidity	1.08	1	0.299
Cultivation phase	67.47	1	**< 0.001**

Survey points nested within complex are also significant for both species, but here only highlight the variation in population size between sites. Based on both regressions and Linear Models it is clear that the factor critical for the occurrences of the two species is the cultivation phase, although *D. suweonensis* reaches peak calling activity later than *D. japonicus* (Fig. 7.4). The fallow and ploughing phases were characterised by a very low calling index for *D. japonicus*, and a single calling male *D. suweonensis*. The tilling phase saw a large increase in calling activity for *D. japonicus*, up to CI = 3, but *D. suweonensis* was still calling at 2.6 % of survey points only.

Dryophytes japonicus calling activity is also significantly influenced by other factors: temperature, pre-seedlings and rain flood (Fig. 7.5). Once CI = 0 excluded, because of its match with the pre-lekking period, the CI increases with the presence of pre-seedlings (n = 55; CI = 1 reaches 7.3, CI = 2 reaches 9.1 % and CI = 3 reaches 20.0 %), and temperature (n = 592, CI = 0 at 15.11 ± 6.29 °C, CI = 1 at 17.26 ± 5.27°C, CI = 2 at 16.52 ± 5.17 °C and CI = 3 at 17.86 ± 3.40 °C). For rain flood, the relation is less intuitive as the number of paddies flooded by rain (n = 19) decreases once paddies are flooded by agricultural water, but the effect is clear as CI = 0 represents 31.6 % of cases, and the three other CI combined represent 68.4 % of cases.

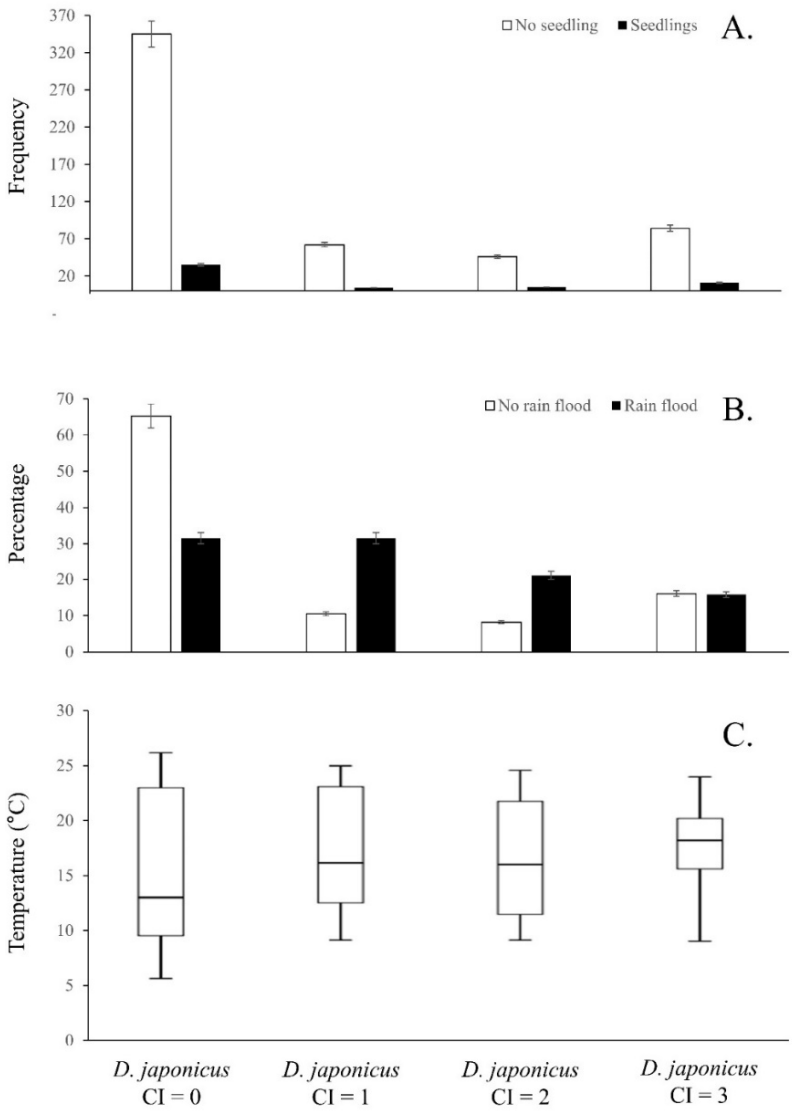


Figure 7.5. When summing the R^2 values from a Pearson's Correlation for each of the variables with the three other variables representative of the presence and activity of *Dryophytes japonicus*, the highest score goes to the CI variable. It is thus used for the graphical representation of the data, here presented for temperature, pre-seedling and rain flood. (A) *D. japonicus* calling in relation to rice pre-seedlings, (B) *D. japonicus* calling index in relation to rain flood, (C) *D. japonicus* calling index in relation to temperature.

Table 7.4. Results of the General Linear Model conducted to assess the significance of environmental and agricultural variables on the CI of *D. japonicus* at sites (n = 592).

	df	χ^2	*F*	*p*-value
Temperature	1	7.34	7.92	**0.005**
Humidity	1	0.01	0.01	0.915
Cultivation phase	4	25.71	27.74	**< 0.001**
Pre-seedlings	1	4.02	4.34	**0.038**
Complex(Site)	39	2.23	2.41	**< 0.001**
Complex	9	1.98	1.46	0.476
Rain flood	1	9.3	10.03	**0.002**
Error	544	0.93		

Seasonal variations in calling activity

Following the definition of the different lekking phases, pre-lekking from replicates 1 to 7, lekking from replicates 8 to 13 and post-lekking for replicates 14 and 15, we found that during the pre-lekking period, (n = 280; from 40 survey point surveyed eight times), there were 0.03 ± 0.24 (mean ± SD) calling male *D. suweonensis* and a median CI of zero for *D. japonicus*. For the lekking period (n = 200), an average of 0.52 ± 1.11 *D. suweonensis* were calling and the median CI was two for *D. japonicus*. Finally, for the post-lekking period, n = 120, a total of 0.14 ± 0.51 individuals *D. suweonensis* were calling, and the median CI for *D. japonicus* was zero again. The median value of CI was zero for all time periods for *D. suweonensis*, due to very low numbers of individuals (Fig. 7.3). During agricultural flooding, the percentage of survey points reaching CI = 3 for *D. japonicus* was at 65.5 %, matching with the transition between the pre-lekking and the lekking period for the species, although *D. suweonensis* was calling at 32.7 % of survey points only. The calling activities of both species peaked at the stage of rice plantation (Fig. 7.4).

Pre- and post-lek

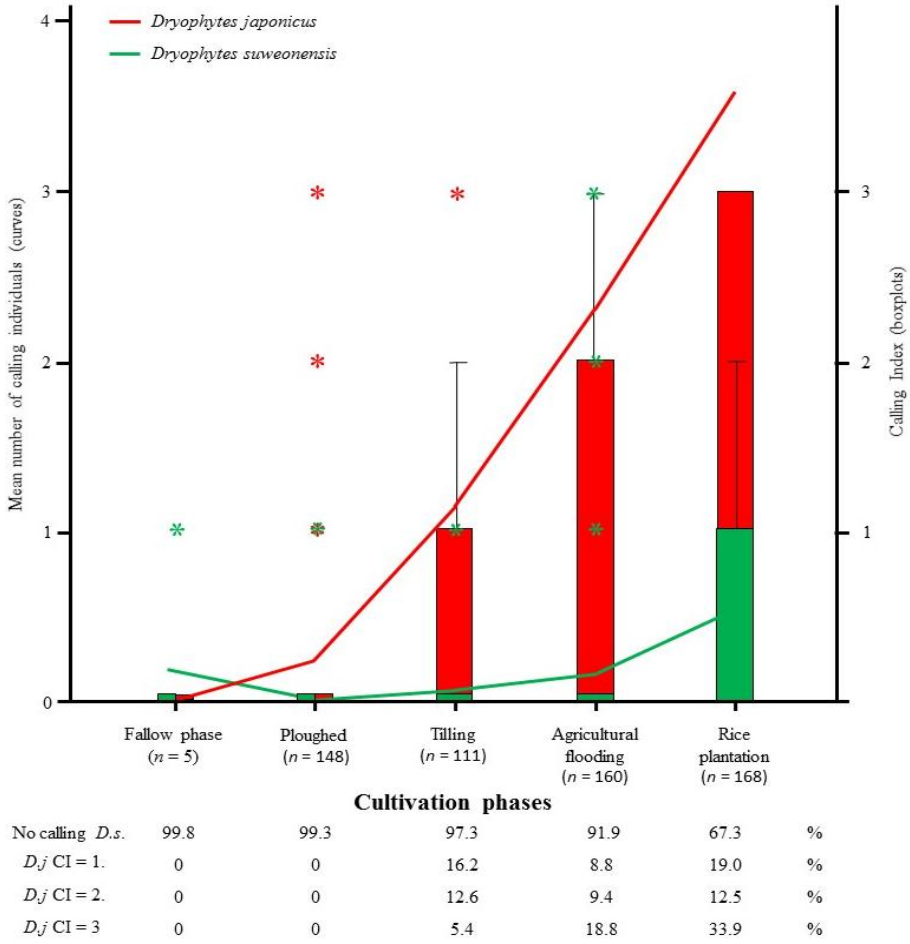
The results of the binary logistic regressions for pre-lekking (Table 7.5) showed that the calling activity of *D. japonicus* was significantly influenced by temperature, while the calling activity of *D.*

suweonensis was significantly related to temperature and season. The binary logistic regressions for post-lekking (Table 7.6) displayed the opposite trend, with *D. japonicus* call production significantly related to agricultural flooding only, while *D. suweonensis* was significantly influenced by season, humidity, and agricultural flooding. For the pre-lekking period, temperature was significant for both species, while flooding was the only significant variable for both species during the post-lekking period. These two variables are limiting factors at these two time periods. The descriptive statistics for the variables significantly influential for the pre-lekking period (Table 7.7) displayed a higher temperature for *D. japonicus*. *Dryophytes suweonensis* calling activity was associated with a higher temperature and the season.

For the post-lekking period, the calling activity of both treefrog species were influenced by a higher ratio of flooded rice paddies. The latter was also related to a lower humidity, and the calling activity also decreased in relation to the season.

Lekking period

The results of the multinomial logistic regression demonstrated a difference between the two species in the factors important for the full lek period. For *D. japonicus*, temperature and cultivation phase were significant, while no variable was significant for *D. suweonensis*.



No calling *D.s.*	99.8	99.3	97.3	91.9	67.3	%
D.j CI = 1.	0	0	16.2	8.8	19.0	%
D.j CI = 2.	0	0	12.6	9.4	12.5	%
D.j CI = 3	0	0	5.4	18.8	33.9	%

Figure 7.4. Relationship between cultivation phase and the number of calling individuals for *Dryophytes suweonensis* (= *D.s*) and CI for *D. japonicus* (= *D.j*). The calling index and sites without calling individuals are in %. Stars denote outliers. The 50 % median for *D. japonicus* is CI = 0 for tilling and agricultural flooding, and CI = 1 for rice plantation. For *D. suweonensis*, the 50 % median is CI = 0 for rice plantation. No whiskers are visible when values are reaching extremes CI values (*i.e.* 1 or 3).

Table 7.5. Binary logistic regression explaining the breeding effort in relation to abiotic variables during the pre-lekking period (surveys 1 to 7). Each species was set as dependent variables and season, temperature, humidity and cultivation phases were set as independent variables (n = 280 for both species).

	Dryophytes japonicus					***Dryophytes suweonensis***				
	B	S.E.	Wald	*Df*	*P*	*B*	S.E.	Wald	*Df*	*P*
Season	0.04	0.02	2.64	1	0.104	0.27	0.12	4.6	1	**0.032**
Temp.	0.33	0.06	25.63	1	**0.001**	0.63	0.22	8.7	1	**0.003**
Humidity	0.02	0.01	2.05	1	0.152	-0.01	0.03	0.01	1	0.971
Cultivation phase	0.23	0.17	1.89	1	**0.169**	-0.11	0.38	0.08	1	0.767

This difference highlights a difference in behavioural plasticity between the two species, potentially correlated with the variation in duration of the breeding season (Table 7.8). Within the significant variables, temperature was at its highest for CI = 1 for *D. japonicus* and two individuals *D. suweonensis* (18.89 ± 3.76 °C, or CI = 1, Table 7.9).

High values for agricultural flooding and tilling were associated with the highest calling index for *D. japonicus*, but the trend was less distinct for rain flooding because of the short seasonality of the variable, and for which the highest values were correlated with CI = 1. The graphical plotting of the variables influential to the calling activity of the two species (Fig. 7.6) shows the close association between calling activity of the species, confirmed through Pearson Correlations (n = 592, R^2 = 0.29, $p < 0.001$), and rain flooding and temperature for the pre-lekking period.

Finally, the Pearson correlation tests for the influence of the weekly calling activity of the two species on each other were not found to be significant ($r < 0.32$; p-value > 0.067), at the exception of week 13 (r = 0.80, p-value < 0.001). Replicate 13 is the last replicate of the lekking period, and we assume that the highest number of calling individuals of both species on that week was related to the optimal breeding conditions, rather than the influence of the two species on each other.

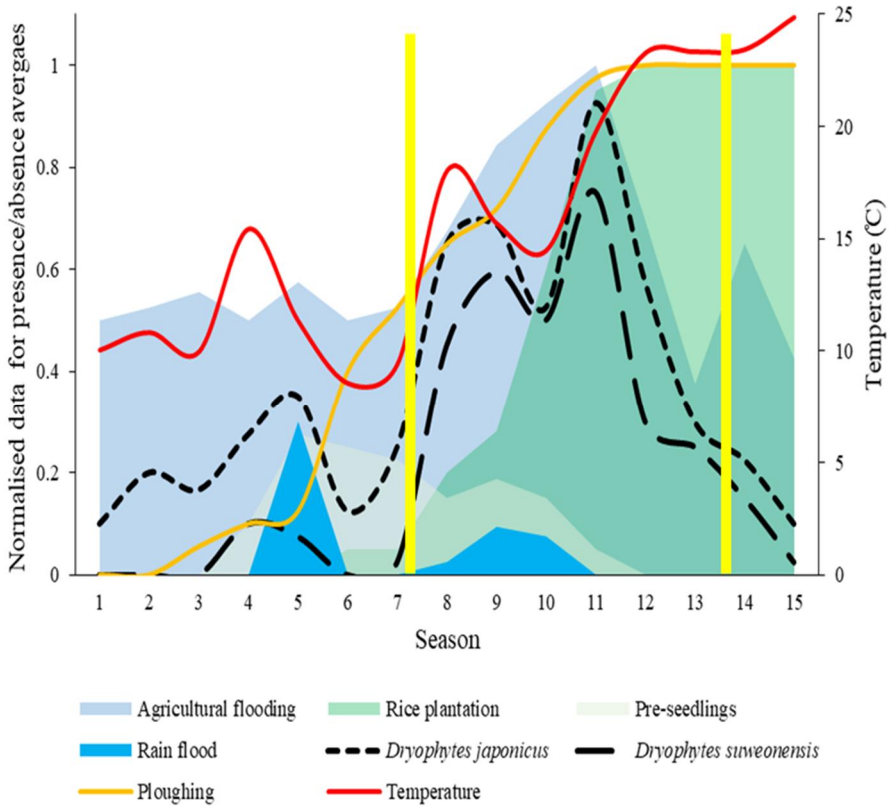


Figure 7.6. Calling activity of both Hylids species in function of all significant agricultural and environmental variables. The first peak in presence for the two Hylid species is due to an overlap between rain floods and pre-seedling, the second peak is correlated with a temperature increase and the last and highest peak is correlated with the overlap of agricultural flooding and rice plantation. The yellow vertical bars denote the lekking period.

Table 7.6. Binary logistic regression explaining the breeding effort in relation to abiotic variables during the post-lekking period (survey 14-15). Each species was set as dependent variables and season, temperature, humidity and agricultural flooding were set as independent variables (n = 120 for both species).

	Dryophytes japonicus					***Dryophytes suweonensis***				
	B	S.E	Wald	*df*	*P*	*B*	S.E.	Wald	*df*	*P*
Season	-0.04	0.02	2.78	1	0.095	-0.05	0.02	5.04	1	**0.025**
Temperature	-0.13	0.26	0.26	1	0.610	0.05	0.26	0.03	1	0.854
Humidity	-0.05	0.05	1.14	1	0.286	-0.12	0.05	5.64	1	**0.018**
Agricultural flooding	-1.55	0.43	13.13	1	**< 0.001**	-0.97	0.41	5.57	1	**0.018**

Table 7.7. Descriptive statistics for pre- and post-lekking for *Dryophytes japonicus* and *D. suweonensis*, respectively surveys 1 to 7 (n = 280) and surveys 14 and 15 (n = 120). "+" stands for "presence and "–" for absence of the species.

		Mean	SD	Min.	Max.	Range
Pre-lekking	***D. japonicus***					
Temperature (°C)	-	10.25	7.35	5.60	16.80	11.20
	+	10.60	6.03	7.50	16.70	9.20
	D. suweonensis	Mean	SD	Min.	Max.	Range
Temperature (°C)	-	10.67	2.44	5.60	16.70	11.10
	+	13.56	2.54	10.00	16.80	6.80
Season (days)	-	10.24	6.76			
	+	13.50	4.22			
Post-lekking	***D. japonicus***	Mean	SD	Min.	Max.	Range
Agricultural flooding (%)	-	29%	46%			
	+	61%	49%			
	D. suweonensis	Mean	SD	Min.	Max.	Range
Agricultural flooding (%)	-	41%	50%			
	+	57%	50%			
Season (days)	-	57.38	10.15	40.00	64.00	24.00
	+	53.00	11.38	40.00	64.00	24.00
Humidity (%)	-	89.76	4.37	78.60	99.90	21.30
	+	87.75	4.14	77.10	97.60	20.50

Table 7.8. Multinomial logistic regression explaining the breeding effort in relation to abiotic variables during the lekking period (surveys 8 to 13; $n = 200$). Each species was set as dependent variables and season, temperature, humidity and cultivation phase were set as independent variables.

	Dryophytes japonicus				*Dryophytes suweonensis*			
	AIC	χ^2	df	*P-value*	AIC	χ^2	df	*P-value*
Season	308.96	1.09	3	0.778	32.65	0.93	3	0.336
Temperature	327.84	19.97	3	**< 0.001**	32.87	1.14	3	0.285
Humidity	311.48	3.62	3	0.688	32.07	0.35	3	0.555
Cultivation phase	404.16	92.69	15	**< 0.001**	38.18	6.45	5	0.265

DISCUSSION

Our results highlight similarities and divergences in agro-environmental preferences between the numerous *Dryophytes japonicus* and the endangered *D. suweonensis*. According to our hypothesis, *D. japonicus* started breeding earlier than *D. suweonensis*, before rice plantation. This matches with the expected behaviour of a species hydroperiodically restrained during its evolution, and thus inclined towards the opportunistic use of water bodies. Oppositely, *D. suweonensis* generally does not start breeding before rice is planted, which corresponds to the expected behaviour of a species that would have been relying on seasonal floods for breeding, over evolutionary times. Adaptation to the two habitats may have also been one of the factors for speciation. As both species reach their peak calling activity once rice seedlings have been planted, the peak calling matches the optimal hydroperiod. However, as *D. japonicus* starts breeding as soon as fields are ploughed, or even earlier if there is rain water partially flooding rice paddies, egg masses may be negatively impacted by subsequent ploughing, tilling and planting.

Although the breeding phenology of both species is clearly linked to agricultural practices, their effects on the two species may appear contradictory with the current population dynamics, as positive for *D. suweonensis* and negative for *D. japonicus*. As a general rule during the whole breeding season, and apart from the agricultural phases, *D.*

japonicus was more sensitive to environmental variations than *D. suweonensis*, a potential artefact of the prolonged breeding period. Generally, the variables important for the breeding activity of *D. japonicus* were temperature and rain flood. Another commonality between the two species is that breeding activity is not linked to humidity, oppositely to common expectations.

The splitting of the breeding period into pre-lekking, lekking, and post-lekking is consistent with the lekking activity described for a number of species (Wells 2010), although the behaviour of female *D. suweonensis* would have to be determined to confirm the match with the definition of lekking species (Ryan 1985; Beehler and Foster 1988). When limited to the pre-lekking period, temperature is important to both species, while season is also important to *D. suweonensis*. The increase in calling activity during the lekking period is remarkable for its superposition with rice plantation, while the decrease in breeding activity is closely linked to the decrease in agricultural flooding after the peak calling activity. The calling activity of *D. japonicus* during the post-lekking period is not related to any variables, and is hypothesised to decreases following a drop in hormonal level (Schmidt 1966; Wetzel and Kelley 1983; Penna et al. 1992). Both season and humidity are important for *D. suweonensis* during the post-lekking period, highlighting the end of the breeding season for the species.

Table 7.9. Descriptive statistics for statistically significant variables for *Dryophytes japonicus* and *D. suweonensis* for the lekking period (surveys 8 to 13; $n = 200$). No CI = 3 were collected for *D. suweonensis*. Cultivation phases are divided into the different categories for ease of understanding. Despite not reaching significance, temperatures are presented for *D. suweonensis* as an indicator.

	CI	Mean	Median	SD	Min.	Max.
Dryophytes japonicus						
Temperature	0	17.44	15.80	4.25	12.20	24.00
	1	19.05	18.90	4.48	12.50	24.50
	2	19.01	19.90	3.69	14.00	23.60
	3	18.47	18.50	2.75	12.60	24.00
Tilling	0	0.78	1.00	0.42		
	1	0.83	1.00	0.39		
	2	0.00	0.00	0.00		
	3	0.98	1.00	0.12		
Rain flooding	0	0.07	0.00	0.25		
	1	0.09	0.00	0.29		
	2	0.00	0.00	0.00		
	3	0.02	0.00	0.12		
Agricultural flooding	0	0.64	1.00	0.48		
	1	0.87	1.00	0.34		
	2	1.00	1.00	0.00		
	3	1.00	1.00	0.00		
Dryophytes suweonensis						
Temperature	0	18.64	18.55	3.89	12.20	24.20
	1	18.16	17.30	3.39	13.10	24.50
	2	17.58	18.50	2.91	12.60	21.20

The shorter breeding season in *D. suweonensis* may partially explain the difference in population sizes between the two species, as *D. japonicus* has more time to deposit eggs and female can lay eggs more than once (Kim 2015a).

In this study, *D. suweonensis* started breeding later than *D. japonicus*, and *D. suweonensis* may require the availability of large pools of water to start producing advertisement calls (Kim 2016). Alternatively, the calling activity of *D. suweonensis* may be triggered by some levels of temperature or photoperiod (Reading 1998; Sugimoto and Jiang 2008), which coincides with the season when farmers start planting rice. From our results, and if such a trigger variable does exist, we can estimate a trigger temperature *circa* 12.87 ± 0.27 °C for *D. suweonensis*. The fact that *D. suweonensis* is now entirely restricted to rice paddies for breeding (Borzée and Jang 2015), and that rice farming may not start as early as natural wetland would be flooded, and thus restraining its breeding potential, is another potential explanatory factor for the endangered status of the species.

Dryophytes suweonensis may benefit from farming activities, which may at the same time negatively impact the first eggs and larvae of *D. japonicus* deposited before agricultural flooding. If these larvae fail to develop, newly hatched *D. suweonensis* tadpoles will not be subjected to competition with older, and more developed *D. japonicus* tadpoles. This is

important for both indirect competition as tadpoles of both species extract the same resources, and for interference competition as tadpoles *D. japonicus* are cannibalistic.

The fact that our results consistently point to a higher number of significant factors for *D. japonicus* than for *D. suweonensis* is hypothetically due to the length of the breeding season. Species with a longer breeding period are generally more dependent on their environment than species that have a shorter breeding season, *i.e.* explosive breeders (Wells 1977; Greene and Funk 2009). Because of a shorter attendance to breeding sites by males *D. suweonensis*, variation in calling activity in relation to the agro-environment may be less detectable than for *D. japonicus*. Additionally, because of the larger number of male *D. japonicus*, more variations can be observed and would highlight the need for a larger sample size for *D. suweonensis* to present increased breeding variability.

The rapid decrease in the number of calling individuals in relation to the agricultural flooding of rice paddies deserves additional research. Although possibly coincidental, it seems that both *Dryophytes* species would benefit from a longer hydroperiod for tadpole development. In case of affluent water resources this would also be beneficial to rice crops as flooding prevents weed growth in rice paddies. This would additionally allow a decrease in pesticide use, which have also been associated with

the decline of *D. suweonensis* (Borzée et al. 2017d). The conservation of *D. suweonensis* seems to require an earlier flooding of rice paddies, and we therefore recommend the flooding of rice paddies during the fallow phase (Machado and Maltchik 2010). This procedure would also benefit the conservation of a number of other species, including the endangered Seoul Golden Frog (*Pelophylax chosenicus*), commonly syntopic with *D. suweonensis*.

Rice paddies are not as biologically diverse as natural wetlands, and neither they are as economically productive as industries or cities. As a result, encroachment is frequent, facilitated by their close proximity to cities, increasing the risks to amphibian's survival (Cushman 2006; Hamer and McDonnell 2008). The low economical value of rice paddies in developed countries is consequently an indirect reason to the rarefaction of amphibian secondary habitats. However, rice paddies are the only breeding ground for some species, and their conservation has to be a priority for numerous species. Finally, an emphasis on the protection of rice paddies as breeding habitat for species is required. We urge the development of farming guidelines for sites where endangered species occurs, under international conservation frames compatible with agriculture, such as RAMSAR.

Chapter eight

SEGREGATED EVOLUTIONARY HISTORY REVEALED BY BRUMATION AND HIBERNATION HABITAT SELECTION OF TWO CLOSELY RELATED TREEFROG SPECIES.

ABSTRACT

Most amphibians are known for migrating between flooded habitats for breeding and dry habitats for non-breeding activities. However, not all species migrate between flooded and dry habitats, and such difference in closely related species may highlight divergent evolutionary history. Through field surveys during brumation and over-wintering periods, field orientation HDF tracking in fall and spring, and laboratory behavioural experiment for brumation and hibernation, we demonstrated differences in seasonal migration and hibernation habitat between *Dryophytes suweonensis* and *D. japonicus*. We found that *Dryophytes japonicus* migrated towards forests for over-wintering, and migrated back to rice paddies for breeding in spring. By contrast, *D. suweonensis* was found to hibernate buried in the vicinity of rice paddies, its breeding habitat. The difference in migrating behaviour was also found to match with variation in microhabitat use during brumation and hibernation between the two species. Our findings highlight different ecological requirements between the two species, and may result from the

segregated evolutionary history of the two species, with speciation potentially linked to the use of a new breeding habitat by a species. Besides, use of rice paddies for both breeding and hibernation may be an additive factor for the endangered status of *D. suweonensis*, due to degradation of hibernation sites in winter.

INTRODUCTION

Multi-environmental life cycles are clear witnesses of evolutionary histories, and reflect acquired breeding strategies in amphibians (Wake, 1982), with aquatic breeding as the shared ancestral character for all amphibians (Reiss, 2002; Schoch, 2009). Thus, migration between breeding and over-wintering habitats is an evolutionary requirement tied to the biphasic life-cycle of the largest number of amphibians (Duellman and Trueb, 1986). The medium used for breeding typically reflects ancestral characters (Duellman, 1989), while migration towards another environment is a more recent evolutionary trait (Semlitsch, 2008). Exceptions do exist, however, such as for plethodontid salamanders, which retain the larvae stage (Chippindale et al, 2004), and for one of the focal hylid species of this study for yet unknown reasons.

Differences in life cycles are evidence of divergence in evolutionary origin between related species (West-Eberhard, 2003; West-Eberhard,

2005). The origin of novel phenotypes stems from the reorganization of ancestral phenotypes, followed by the genetic accommodation of changes (Mayr, 1963; West-Eberhard, 2005). Selection acts on phenotypes (Mayr, 1963) and thus populations subject to differential environmental pressure may see the apparition of specific phenotypes, that are subsequently integrated into genotypes (West-Eberhard, 2005). This is for instance the case of tropical vines *Monstera* sp. displaying varying leaf forms, and resulting in species-specific ontogenies (Madison, 1977), but also the case of role-reversed sandpipers species where males incubating eggs show the same increase in prolactin as incubating females (Beach, 1961; Oring et al, 1986). Another example is the interspecific variations in the expression of parental care due to ecological requirements in Microtus spp. (West-Eberhard, 2003).

Seasonal migration is only one type of population displacement among several (Semlitsch, 2008), but it is the most common non-circadian migration, seen from whales (Kenney et al, 2001) to butterflies (Brower, 1995). Seasonal migration is also common in amphibians (Sinsch, 1990; Ryan and Semlitsch, 1998); for instance, *Bufo bufo* hibernates in terrestrial hibernaculum (Van Gelder et al, 1986) and migrates to water bodies to breed in spring (Gittins, 1983). Some amphibians species will migrate considerable distances away from their breeding site to find shelter against climatic variations (Griffiths 1984), and 15 km is considered the limit to

direct migration and dispersion due to physiological requirements (Sinsch, 1990). A representative assessment of eight Central European amphibian species demonstrated migration distances between *circa* 100 and 2200 m from the breeding site (Kovar et al, 2009). Other species, such as *Lithobates catesbeianus*, hibernate in the vicinity of their breeding site, and thus do not require to migrate (Stinner et al, 1994). However, there is still no information about migration for a large number of species. For instance, when focusing on Hylidae, field observations are the most reliable data for European species (reviewed by Stumpel, 1990), some behavioural ecology studies are published for North American spcies (Mahan and Johnson, 2007; Johnson et al, 2008). Some Indian species are known to shelter from the cold in banana stems (Iangrai, 2011). *Dryophytes japonicus* in North East Asia is comparatively well studied and known to start hibernating because of the rise of *cirp* RNA due to cold and photoperiod (Sugimoto and Jiang, 2008). The species is able to withstand massive temperature drops, down to -53°C in laboratory settings (Berman et al, 2016), and to principally use forested hills for hibernation (Borzée et al, *in review-a*).

In amphibian species in the Republic of Korea, both aquatic and dry types of non-freeze-resistant hibernation types are known. For instance, *Rana spp.* can hibernate under water (Lee and Moon, 2011; Macias et al, in review), and are the first species present at the breeding

sites after ice thaw (Yoo and Jang, 2012). Oppositely, *Dryophytes japonicus* hibernates under decaying vegetation on hills forested by oak trees principally (Borzée et al, *in review-a*). It is unclear if the other treefrog species from the peninsula, the endangered *D. suweonensis*, can follow the same pattern. Unlike *D. japonicus*, the species is not found in forests during the non-breeding season. Furthermore, there are anecdotal observations of *D. suweonensis* hibernating in the banks of rice paddies (Pers. Comm. Kim Hyun-Tae). *Dryophytes suweonensis* is known to have originally bred in low altitude alluvial wetlands, although now restricted to rice paddies (Borzée and Jang, 2015), while *D. japonicus* breeds in a much wider range of environments, as long as solid substrate is available for call production (Borzée et al, 2016a). Besides, the two species display microhabitat segregation during the production of advertisement calls, likely due to competition (Borzée et al, 2016a). Besides, due to the long list of traits linked to the breeding behaviour of the species (Roh et al, 2014; Borzée and Jang, 2015; Borzée et al, 2015a; Kim, 2015; Borzée et al, 2016b; Kim, 2016; Borzée et al, 2017), we hypothesize that *D. suweonensis* hibernates in rice paddies, where it also breeds, while *D. japonicus* is expected to migrate seasonally between breeding sites and forests hills, where it hibernates. Here, we tested this hypothesis through winter field surveys of the two *Dryophytes* species. The absence of seasonal migration may indicate that the two species share the same

breeding and overwintering habitats, and thus exploit the same environment, while differences in seasonal migrations would suggest a different evolutionary history and the use of different environments.

MATERIAL AND METHODS

This project is composed of five experiments for both Dryophytes suweonensis and *D. japonicus*: (1) field observations for brumation, (2) field orientation tracking for brumation, (3) laboratory brumation and hibernation observations, (4) winter field observations and finally, (5) spring orientation tracking. The chronological numbering is from pre-hibernation, to emergence from hibernation. All experiments were conducted with the agreement of the Ministry of Environment from the Republic of Korea, under the permits numbers: 2013-16, 2014-04, 2014-08, 2014-20, 2015-3, 2015-4, 2015-6, 2015-28, and 2016-5.

(1) Field observations for brumation

Observations of the brumation ecology of the two species in the field were collected at two localities in 2014 (n = 29) and four localities in 2015 (n = 32; Fig. 8.1). The two localities from 2014 (# 1 and 2; Fig. 8.1) are included within the four localities from 2015 (together with remaining sites # 3 and 4; Fig. 8.1). The locality 1 is composed of one paddy site and two forested sites, while the localities 2, 3 and 4 are each composed of one

paddy site and a single forested site. The localities were selected following observations of calling males produced by both *Dryophytes* species during the breeding season (see Borzée and Jang, 2015).

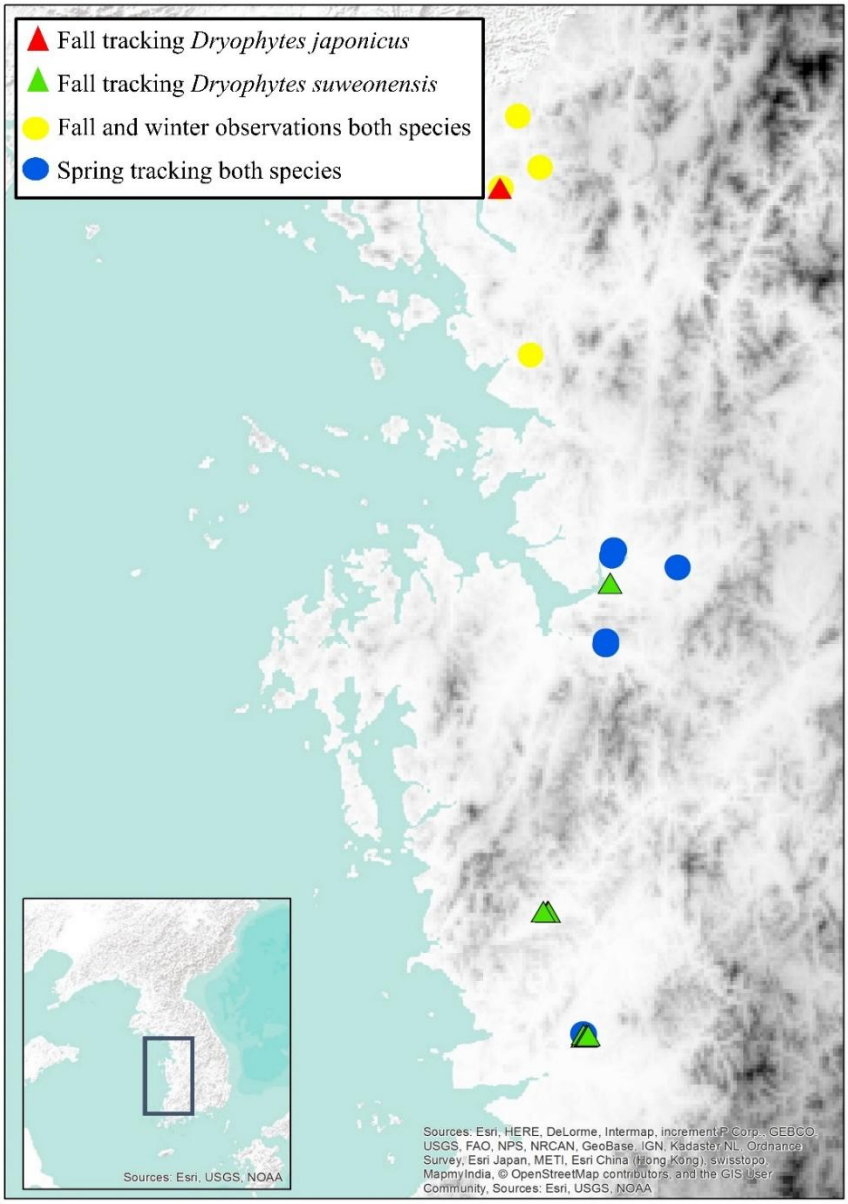


Figure 8.1. Spatial location of sites surveyed in this study. This map was generated with ArcMap 9.3 (Environmental Systems Resource Institute, Redlands, California, USA; http://www.esri.com/).

Surveys were initiated in September on both years and terminated after the first freeze. In 2014, the sites were surveyed the 1st and 3rd week of September, every week of October and the 1st week of November. In 2015, all sites were surveyed on the 3rd week of September, 1st and 3rd week of October and 1st week of November. The surveys were conducted through spotlight line transects (Smith and Nydegger, 1985), where the researcher follows a pre-determined 250 m-long transect and visually inspects the vegetation for individuals.

All transects at rice paddies site were conducted for varying distances, along the cemented straight road (Fig. 8.2) bordered by a row of planted beans on at least one side, favoured by the two species (Borzée and Jang, 2017). Transects at forest sites were conducted along a 250 m approximatively straight line due to the topology of the field. Each hylid individual found was hand caught and the species was identified based on morphology (Borzée et al, 2013).

Tracking general (methodology partially shared for 2 and 4)

For all microhabitat use and directionality experiments in this study, we used a Harmonic Direction Finder (HDF; R2 RECCO AB; Lidingö, Sweden), relying on a passive dipole attached to the individual to be tracked with a gauze waist band. The HDF emits microwaves towards the dipole, which bounces it back through an antenna tailored for each

individual, and encodes for directionality and distance (Pellet et al, 2006; Pašukonis et al, 2014; Borzée et al, 2016a). We soldered a schottky diode (model R2 RECCO AB; Lidingö, Sweden) on a tin-platted copper wire folded on itself at 180 degree in a fashion that creates a loop 1 cm away from the bent, prolonged by two isolated segments of the wire, resulting in an antenna. This design maintains the electric properties of the diode and provides mechanical elasticity of the antennae. The Stocky diode reflects the wave received at twice its frequency (see de Moura Presa et al, 2005 and Borzée et al, 2016a for details), which is then translated by the HDF into an acoustic signal of varying intensity in function of the direction and distance to the dipole, and thus allowing for the localisation of the organism bearing the antenna. To isolate the electrical dipole, the antennae were uniformly insulated with a silicone spray (NABAKEM, S-830 UL94 V-0, Seoul).

We selected the initial antennae with two 8-cm legs, resulting in *circa* 25 m effective range. The waistbands to which the antennae were attached were prepared with varying length for each individual, to ensure that the antennae weight was below the recommended 5 % of each individual's body mass.

(2) F**ield orientation tracking for brumation**

Tracking during brumation was conducted separately for the two treefrog species. HDF tracking was conducted in 2013 for *D. japonicus* as

the species was already known to be hibernating on forested hills (Sugimoto and Jiang, 2008; Borzée et al, *in review-a*). However, the first anecdotal observation for the overwintering behaviour of *D. suweonensis* was collected in 2015, following which we conducted brumation tracking.

Tracking was conducted for 24 h for each frog, and individuals were released at least 15 m from each other to prevent detection overlap. For each tracking point, a colour flag was placed *circa* 10 cm from the frog to measure the displacement and the directionality of the movement between successive points. Every hour temperature (°C), luminosity (lux), relative humidity (%), height from the ground (cm), distance moved from the previous point (cm) and type of microhabitat were recorded. The types of microhabitats were "grass", "rice", "buried", "ground" and "bush".

We also annotated the directionality of the movement, towards either the centre of the forest or the centre of the adjacent rice paddies. The movement was decomposed in the form of a vector, for instance, 50 cm towards the forest and 20 cm towards the rice paddies. All measurements were taken at a 5-cm resolution as not to overly disturb the frogs.

Dryophytes japonicus

For this experiment, the waistband to attach the antenna to the frogs was made of gauze sewed on itself ventrally, and thus adjusted for

the size of each individual.

Figure 8.2. Example of the brumation and winter field observations at the site #1: "Sihung", located 37.406° N and 126.805° E. The red lines represent the transects, in the rice paddies on the west and in the forests in the East. The red star indicates the sites were the hibernating female was found during the winter field observations. This map was generated with Google Earth Pro (Google Earth imagery, v7.1.2.2041, 2013), on maps from 2016 SKEnergy, Image Landsat Copernicus.

Tracking was conducted for nine males and one female *D. japonicus* in the city of Paju (red marker; Fig. 8.1), on September 27 to 30, 2013. Individuals had been previously caught in the forest adjacent to the rice paddy complex (see Borzée et al, *in review-a*) and raised for one month under controlled conditions in the lab. Each individual was released at the edge of a rice paddy, between 174 and 209 m away from the edge of the forest where they had been caught. Individuals were tracked for 2328.33 ±719.63 min on average, resulting in an average of 125.98 ± 17.70 (mean ± S. D.) min interval between tracking points, for a total of 172 observations.

Dryophytes suweonensis

For this experiment, each waistband was made of silicone tubing (diameter = 1.8 mm) within which was inserted the loop of the antennae. The loop was connected ventrally by electrical paint (BareConductive 10 ML, Bare Conductive Ltd; London, UK) and thus working as an electric dipole while tailored for the size of each individual. We conducted tracking for four individuals of each sex at four independent sites over a 110 km range (green markers, Fig. 8.1). Tracking was conducted between October 8 and 16, 2015. Individuals had been caught *circa* one month earlier at the same site (Kim, 2016; Borzée et al, *in review-b*) and had been raised in controlled conditions in laboratory since then. Each

individual was released at the edge of a rice paddy, between 139 and 1018 m away from the edge of the closest forest. Individuals were tracked for 1332 ± 120 min on average, for a total of 305 observations.

(3) Laboratory brumation and hibernation observations

Two experiments were conducted in laboratory conditions for both species, the first one on microhabitat selection during brumation, referred to as "lab brumation", and the second one on microhabitat selection during hibernation, referred to as "lab hibernation". For both species, individuals originated from egg masses collected from the wild (Kim, 2016; Borzée et al, *in prep*), and were kept in the lab from hatching to release (permit 2015-4 issued by the Ministry of Environment of the Republic of Korea. Five individuals from five egg masses were collected at five different locations (blue markers, Fig 1; n = 25 for each species), and tadpoles were raised in independent PCV aquariums (20 x 30 x 20 cm; W x L x H) for this experiment. After metamorphosis, individuals were isolated, providing a sample size of five individuals raised independently for each of the five families for each species. Each metamorph was raised in a glass terrarium 45 x 45 x 45 cm with lateral opening, transparent sides, and screen top (PT2605, Exo-terra, Hagen, Montreal, Canada).

All terraria were set with wet towels at the bottom, which were changed weekly or more often if needed. A non-glazed terra-cotta conic

pot (25 × 16 cm; diameter × height) was set laying horizontally at the back-left of the terrarium. A water filled glazed water-dish (3 cm deep, 12 cm diameter) was set on the front-right, with a 5 x 3 cm non-glazed terra-cotta cylinder pot set upside down within the water dish. This setting allows for terra-cotta pots to absorb water and release it through evaporation within the terrarium to keep the humidity relatively constant (48.48 ± 11.18 % rH). Finally, a wooden cylinder (2.5 cm diameter) was set from the bottom front-left corner to the top back-right corner. Oak was selected due to known preferences from *D. japonicus* (Borzée et al, *in review-a*). The terraria were set onto two four-layered shelves, with their position randomised every second week. Each terrarium was illuminated by its own lighting (UV-B bulbs), and we estimated that the position of the terrariums did result in any bias in the experiments.

Each of the 50 terraria was sprayed daily and the water dish refilled *ad libitum* with carbon filtered and 72-h evaporated water. The frogs were set on a circadian cycle matching with the natural one, readjusted weekly, under natural spectrum illuminations. Crickets were the main diet items used to feed the frogs, but fruit flies and maggots supplements were also given when available. All prey items were powdered with calcium and multivitamins prior to use.

For both brumation and hibernation experiments, the position of the individual, *i.e.* substrate use, and its height from the bottom of the

terrarium, were recorded three times a day (variable named "time period"). We also collected date, time, temperature (°C) and relative humidity (%) for each survey point. Readings of temperature and humidity were noted once for all 50 terrarium replicates due to their setting in a common rearing room exposed to controlled climatic variations following natural variations (see Borzée et al, *in review-b* for details). There were five categories of microhabitat in each aquarium: big-pot (on or within the large terra-cotta pot, representative of sheltering behaviour), ground (if anywhere on the paper towel, representative of ground microhabitats), wood (if sitting on the wood cylinder, representative of perching behaviour on wooden microhabitats, as known to be important for *D. japonicus* brumation, Borzée et al, *in review-a*), pot in water (representative of flooded or damp habitat, as known to be important for *D. suweonensis* brumation, earlier part of this study), and glass (when an individual was holding on a glass panel, not representative of any wild habitat but encoded to prevent any bias in for further analysis). Whenever an observation was made, the use of microhabitat was noted.

The brumation experiment was conducted between September 23 and October 16, 2015, leading to a total of 1906 observations. The hibernation experiment was conducted between December 1 and 23, 2015, leading to a total of 1612 observations.

(4) Winter field observations

The purpose of this set of observation was to observe frogs of both species hibernating in their natural environments. The four sites where brumation observations were conducted were visited again on the 3rd week of January 2015 to look for buried individuals. A total of 4 h was spent at each of the four sites digging the soil down to 50 cm deep in the areas where the frogs of the two species were last seen.

(5) Spring orientation tracking

The frogs used for this experiment were the ones raised from hatching and used for the two lab experiments. They were maintained in the setting described above until tracking, and were released after the spring orientation tracking experiment. The sites for the experiment aiming at determining the post-hibernation behaviour of the two treefrog species were distributed along the whole range of *D. suweonensis* (blue markers, Fig. 8.1). The sites were the same as the ones where egg masses were collected. The tracking procedure in the spring tracking experiment was the same as that of the fall tracking for *D. suweonensis*.

Field work was conducted between May 25 and June 17, 2016, for a total of 20 individuals from each species. Ten individuals of each species were released in rice paddies and the remaining 10 in forested areas at the edge of rice paddies. The forest is composed of Chinese chestnuts (*Castanea spp.*) and pine trees (*Pinus spp*) where *D. japonicus*

is typically found during the non-breeding season (Borzée et al, *in review-a*). The position of each individual was checked every hour and we took note of date, time of day, GPS coordinates, vegetation type, temperature, humidity, height, total movement, movement towards rice paddies and forest. The directionality was assessed based on the movement towards the selected landscape features. An average of 22.07 ± 2.32 h (n = 25 for each species) was spent tracking per individual (duration ± SD). As all individuals were tracked for over 16 h, all were included in the subsequent analyses. The microhabitats recorded were grass, rice, buried, ground and bush. A maximum of five individuals was tracked at the same time, while avoiding tracking individuals within 50 m of each other to prevent overlap in HDF detection signal. Each individual was released between 20:00 and 00:00 to prevent increased predation due to the tracking apparatus.

Data analysis

After the tracking experiments, we mapped the GPS coordinates for all tracked points for each individual on Google Earth (Google, California, USA). For each data point, we then measured the directionality of the movement, such as the angle between the forest, the tracking position of the individual and its subsequent tracking position. Here, the forest was defined as a point situated at the gravity centre of 10 points randomly chosen on the edge of the forest. As all individuals were released at different points and their orientation towards landscape

elements was of prime interested, versus directionality towards cardinal points, 0 degree was defined as towards the forest. The decision to set the forest as 0 degrees was an arbitrary decision made for ease of graphical interpretation and because individuals rarely expressed a total change (i.e. 180 degrees) in directionality. Thus, an angle between 270 and 90 degrees represented a displacement factoring positively towards the forest and an angle of 0 degrees was a straight line towards the forest, and an angle of 180 degree was a straight line away from the forest. All angles were measured on screen with the software imageJ (National Institutes of Health, Maryland, USA). For ease of analysis for models assessing directionality of migration patterns but not involving interspecies comparisons, the directionality was binary encoded as towards or away from forest. Variations in directionality through angles were analysed through circular statistics separately, when required.

(1) To assess seasonal variations in occurrence for both species, and compare the occurrence patterns between the two species, we first analysed the dataset through a repeated-measure ANOVA. To do so, the repeated surveys, continuous variable hereafter defined as “season”, were set as the dependent variable with seven levels, corresponding to the seven surveys replicates, while occurrence for *D. suweonensis* and *D. japonicus* were set as factors. To run this analysis, we tested for homogeneity of variance with Levene’s test, and as the error variance was not equal across

groups for the 6th replicate only, we ignored the partial violation of assumption for the statistical analysis. We also tested for sphericity assumption with Mauchy's test, and ran the repeated measures ANOVA with the Greenhouse-Geisser correction (Scheiner and Gurevitch, 2001) due to the violation of the assumption of sphericity. Furthermore, compound symmetry (homogeneity of the variance-covariance matrix) was assumed for this analysis. We then graphically matched the variations in occurrence over weeks in relation with the two species and the type of site with the results of the statistical analysis.

(2) Due to the lack of correspondence between the data for *D. japonicus* and *D. suweonensis*, the data for each species was first analysed in relation with directionality to forest, and then compared. The fall tracking for *D. japonicus* was first tested for the significance of directionality towards forests for individuals. As data were either temporally or spatially independent, the directionality variable was set as dependent variables in a binary logistic regression. The last assumption was also met as the individual variable, set as the independent variables was on a nominal scales.

Once directionality established, we analysed the dataset through a univariate GLM to find the factors important to directionality. Thus, directionality was set as dependent variable, encoded such as towards paddies, towards forests, for no movement distance travelled as fixed

factor; frog ID, day, time of day and microhabitat as random factors and temperature and luminosity as covariates under a main effect model. After visually testing for the absence of outliers through the analysis of box-plots, we determined the normal distribution of the data with the Kolmogorov-Smirnov test for normality with Lilliefors Significance Correction ($0.14 \leq D_{(151)} \leq 0.51$, $p < 0.001$, and determined the homogeneity of variance with Levene's test for homogeneity of variances ($F_{34,116} = 2.19$, $p = 0.001$). Relative humidity was not included in the analysis due to its correlation with temperature (Pearson Correlation, $R = -0.16$, $p = 0.044$). We subsequently explained all significant variables through descriptive statistics.

The fall tracking for *D. suweonensis* was analysed in a similar way as the one for *D. japonicus,* fist testing for the significance of directionality towards forests for individuals. As data were either temporally or spatially independent, the directionality variable was set as dependent variables in a binary logistic regression. The last assumption was also met as the individual variable, set as the independent variables was on a nominal scales.

We then analysed the dataset through a GLM to find the factors important to directionality. For this model, directionality was set as the dependent variable, distance travelled as a fixed factor; site, sex and vegetation as random factors and date, time of day, temperature, height,

luminosity and frog ID as covariates, under a main factor model. After visually testing for the absence of outliers through the analysis of box-plots, we determined the normal distribution of the data with the Kolmogorov-Smirnov test for normality with Lilliefors Significance Correction ($0.07 \leq D_{(301)} \leq 0.47$, $p \leq 0.001$, and determined the homogeneity of variance with Levene's test for homogeneity of variances ($F_{(136,164)} = 4.28$, $p < 0.001$). In this analysis as well, relative humidity was not included in the analysis due to its correlation with temperature (Pearson Correlation; $R = -0.81$, $n = 304$, $p < 0.001$). The significant variations were then explained through descriptive genetics.

Finally, to understand the difference in movement patterns between the two species, the angles described by the movement in relation with forests were also analysed through a circular 2-samples geometrical directional analysis. As the data were temporally and spatially independent, but not meeting the prerequisite assumption of von Mises distribution (Watson´s U2 test; $U2 = 1.72$, $p < 0.005$; Lockhart and Stephens, 1985), a non-parametric Mardia-Watson-Wheeler (Mardia, 1972) test was used, with angle as dependent variables and species as independent variables. The analysis was ran under an axial (orientation) model. This additional test was conducted despite experiments being conducted at different years and sites for the two species, the results were used to link the two analyses and highlight the difference between the two

species.

(3) The lab experiment on microhabitat use for brumation was analysed first through a Multinomial Logistic Regression to detect variations in microhabitat use between the two species. Thus, microhabitat was set as dependent variable, and the independent variables were species, family, and individual ID nested within family as factors, and temperature, height, time, and date as covariates. Humidity was not used in the model due to the correlation with temperature ($r = 0.40$, $p < 0.001$) and date ($r = 0.11$, $p = 0.001$). The regression was ran under a main effect model. A Multinomial Logistic Regression was selected in agreement with the fulfilment of assumptions. No outliers were detected through the analysis of box-plots. There was a linear relationship between the continuous independent variables and the logit transformation of the dependent variable, tested through the Box-Tidwell (1962) procedure with Bonferroni corrections (Tabachnick and Fidell, 2014), with $p > 0.379$ for all variables and thus rejecting the null-hypothesis. We then described the variations between the different microhabitat and other significant results.

The lab experiment on microhabitat use for hibernation was analysed the same way as for the brumation period as the experiment was conducted in the same experimental setting and the same data were collected. The Multinomial Logistic Regression was also ran in agreement with assumptions: no outliers were observed through the analysis of box-

plots, humidity was not used in the model due to the correlation with temperature ($r = 0.09$, $p < 0.001$) and date ($r = 0.08$, $p = 0.001$) and there was a linear relationship between the continuous independent variables and the logit transformation of the dependent variable with $p > 0.116$ for all variables.

As the same variables were significantly different for the two species for both phases of the experiment, as tested above, we proceeded to run an additional Multinomial Logistic Regression to assess whether the two species differed in microhabitat use between the two phases of the experiment (*i.e.* brumation and hibernation). To do so, microhabitat was set as dependent variables and for the independent variables, phase and species were set as factors and height as covariates. For assumptions requirement, no outliers were detected, no variables were significantly correlated and there was a linear relationship between the continuous independent variables and the logit transformation of the dependent variable with $p > 0.358$ for all variables.

(4) Due to the low number of individuals found during the field observations for hibernating individuals, we could not conduct any statistical analysis and the results only are descriptive.

(5) To determine microhabitat preferences and directionality post hibernation for the two species, we first assessed the correlation between

variables to avoid collinearity in subsequent analysis. We detected significant correlation between total displacement and displacement towards paddies (Pearson Correlation; $R = 0.20$, $n = 873$, $p < 0.001$); and between total displacement and displacement towards forest (Pearson Correlation; $R = -0.26$, $n = 873$, $p < 0.001$). The variables ID, date (Pearson Correlation; $R = 0.95$, $n = 873$, $p < 0.001$) and sites (Pearson Correlation; $R = 0.96$, $n = 873$, $p < 0.001$) were also correlated. Finally, temperature and humidity followed the same trend (Pearson Correlation; $R = -0.79$, $n = 873$, $p < 0.001$). Out of the correlated variables, only one of each group was included in subsequent analyses.

We then ran a GLM to determine the differences in directionality between the two species post-hibernation. Thus, binary encoded directionality was set as dependent variable, species and ID as fixed factors, habitat as a random factor, and date, time, temperature and height as covariates, under a main effect model. A few variables were not included in the model because of collinearity: humidity was correlated with temperature (Pearson correlation test; $r = -0.78$; $n = 873$; p-value < 0.001), total displacement was correlated with directionality (Pearson correlation test; $r = 0.33$; $n = 873$; p-value < 0.001), and site type with microhabitat (Pearson correlation test; $r = -0.41$; $n = 873$; p-value < 0.001). A GLM was selected after visually testing for the absence of outliers through the analysis of box-plots, we determined the normal

distribution of the data with the Kolmogorov-Smirnov test for normality with Lilliefors Significance Correction ($0.14 \leq D_{(151)} \leq 0.51$, $p < 0.001$, and determined the homogeneity of variance with Levene's test for homogeneity of variances ($F_{(68,804)} = 6.03$, $p = 0.001$). The significant differences were highlighted by descriptive statistics, additional *T*-tests and circular statistic tests when *ad-hoc* analyses was required. Due to significant difference in directionality between the two species, we analysed the differences in displacement angle through a suite of circular 2-samples geometrical directional analyses, two-by-two for the variables species, release habitat and angles. As the data were temporally and spatially independent, but not meeting the prerequisite assumption of von Mises distribution (Watson´s U2 test; U2 = 2.65, $p < 0.005$), a non-parametric Mardia-Watson-Wheeler test (Mardia, 1972) was used, with angle as dependent variables and either species or release habitat as independent variables. The analyses was ran under an axial (orientation) model. Biostatistical analyses were computed using SPSS v 21.0 (SPSS, Inc., Chicago, IL, USA) while circular statistics were conducted under PAST v 3.17. (Hammer et al, 2001).

RESULTS

(1) Brumation field observations

The field surveys during the brumation period highlighted a clear

difference in habitat preference between the two species: during brumation, *D. suweonensis* was present at rice paddies only while *D. japonicus* occurred at both rice paddies and forests (Fig. 8.3).

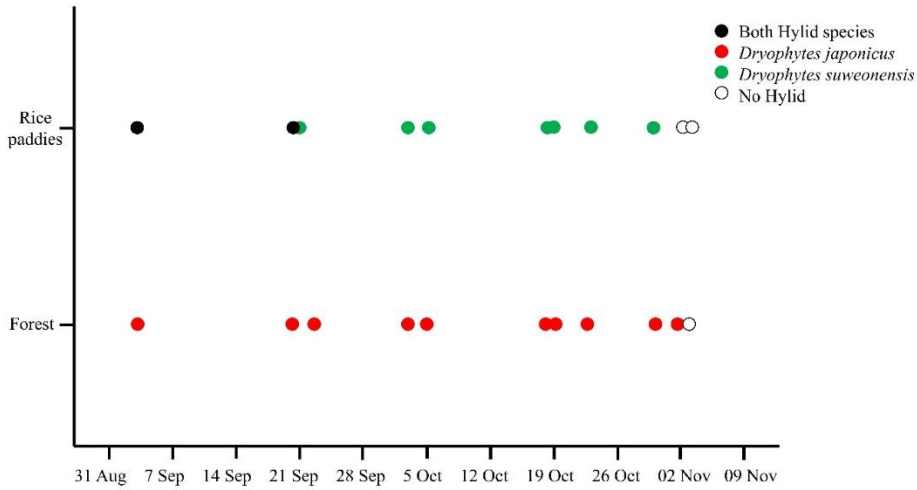


Figure 8.3. Difference in habitat use by the two *Dryophytes* species during the brumation period, as recorded during field observations (1) at two localities in 2014 (n = 29) and four localities in 2015 (n = 32. *Dryophytes japonicus* is present at both rice paddies and forests until late September, before habitat preferences diverged between the two species.

However, *D. japonicus* was present in rice paddies until the last week of September only, from which date onwards, the species was only seen in forests.

None of the species was detectable by spotlight transects from the last week of October, temporally matching with the first freeze. The results of the repeated-measure ANOVA (n = 61) supported these, showing that the occurrence of *D. suweonensis* significantly varied with season (χ^2 = 0.64, d = 6, $p < 0.001$), and *D. japonicus* followed the same pattern (χ^2 = 0.58, d = 6, $p < 0.001$). Thus, our results highlight a significant variation in habitat use: at the beginning of the surveys, both species were in rice paddies, while during the week preceding hibernation, *D. suweonensis* was in rice paddies and *D. japonicus* was in forests.

***(2) F**ield orientation tracking for brumation*

During the fall tracking (2) experiment for *D. japonicus* in 2013, there was a clear directionality pattern. The species moved away from the rice paddies in 63.6 % of cases, and towards the forest in 66.2 % of cases, while moving towards paddies in 36.4 % of cases, and away from forests in 33.8 % of cases. Here, away from paddy and towards forest conveys the same directionality vector, but the percentages are not equal to 100 % if cumulated two by two as the directionality is divided into vectors that can have either 1 or 2 several constituents

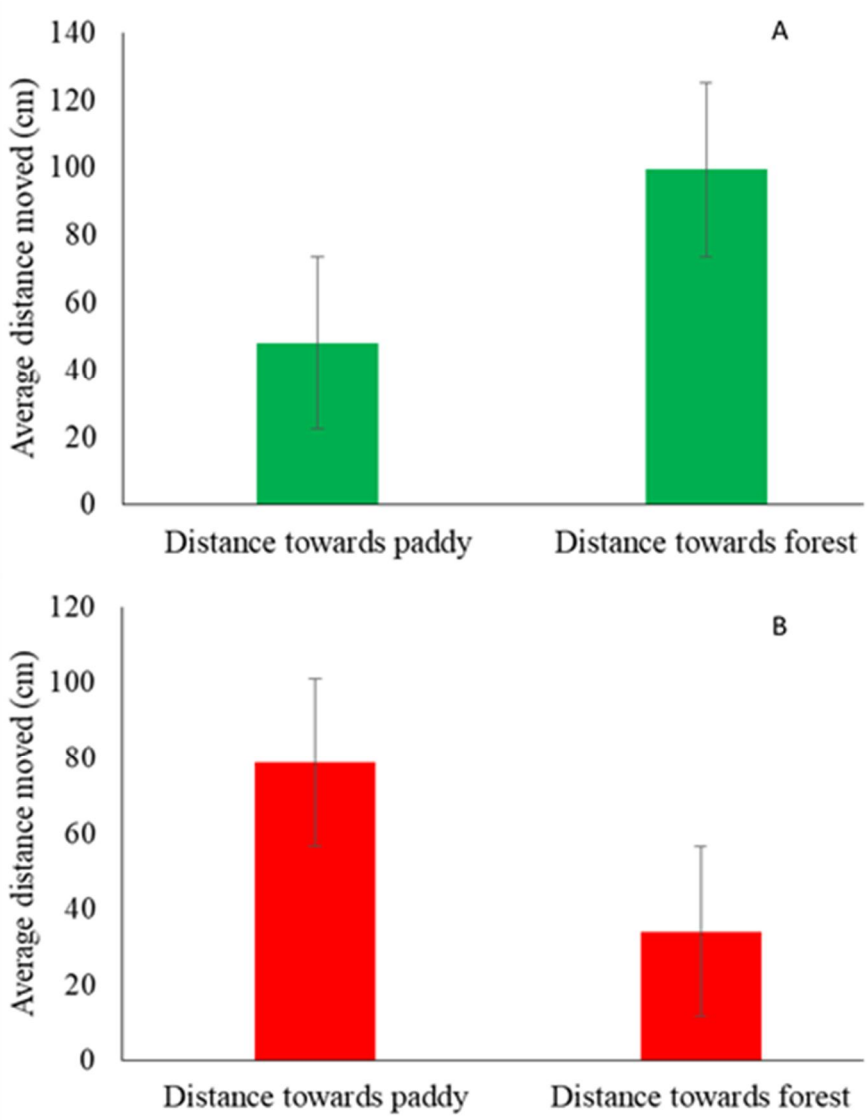


Figure 8.4. Average distance moved per time period for *Dryophytes japonicus* (A) and *D. suweonensis* (B). The distance drawn is shown as positive for ease of understanding, as the non-corrected distance towards forest was negative for *D. suweonensis*.

Table 8.1. GLM to test the relation between directionality and other factors collected during the tracking experiment to investigate the brumation behaviour of *Dryophytes japonicus.*

		Type III - $\Sigma\chi^2$	*df*	χ^2	*F*	*p*-value
Distance travelled	Hypothesis	13.95	24	0.58	30.47	0.032
	Error	0.04	2	0.02		
Individual ID	Hypothesis	1.84	8	0.23	12.09	0.079
	Error	0.04	2	0.02		
Day	Hypothesis	0.52	2	0.26	13.52	0.069
	Error	0.04	2	0.02		
Time	Hypothesis	16.90	100	0.17	8.86	0.107
	Error	0.04	2	0.02		
Habitat	Hypothesis	0.39	1	0.39	20.69	0.045
	Error	0.04	2	0.02		
Temperature	Hypothesis	0.03	1	0.03	1.83	0.309
	Error	0.04	2	0.02		
Luminosity	Hypothesis	0.01	1	0.01	0.48	0.559
	Error	0.04	2	0.02		

. The logistic regression model was statistically significant, $\chi^2_{(1)} = 8.38$, $p = 0.004$. The model explained 78.0 % (Nagelkerke pseudo-R^2) of the variance in directionality and correctly classified 58.9 % of cases.

The grass and bush microhabitats were used by *D. japonicus* to move towards paddies in 1.8 % of cases, while the rice microhabitat was used in 96.4 % of cases. Most movements towards forests were also done in rice (94 %), while remaining movement towards forest was in grass (4%) and bush (2%). The distance travelled in relation to directionality was also significantly different between the two species (Table 8.1), with an average distance moved towards the rice paddies of 48.10 ± 7.8 cm, and an averaged distance moved towards the forest of 99.4 ± 11.2 cm (Fig. 8.4). ID, day, time of day, temperature and luminosity were not significant factors to directionality (Table 8.1).

During the fall tracking experiment (2) for *D. suweonensis* in 2015, there was no clear difference in directionality, in opposition to *D. japonicus* (Fig. 8.5). The species moved away from the rice paddies in 3.0 % of cases while moving towards paddies in 41.14 % of cases, and away from forests in 20.4 % of cases and towards forests in 16.4 % of cases. The percentages are not equal to 100 % if cumulated two by two as the directionality is divided into vectors that can have either 1 or 2 several constituents.

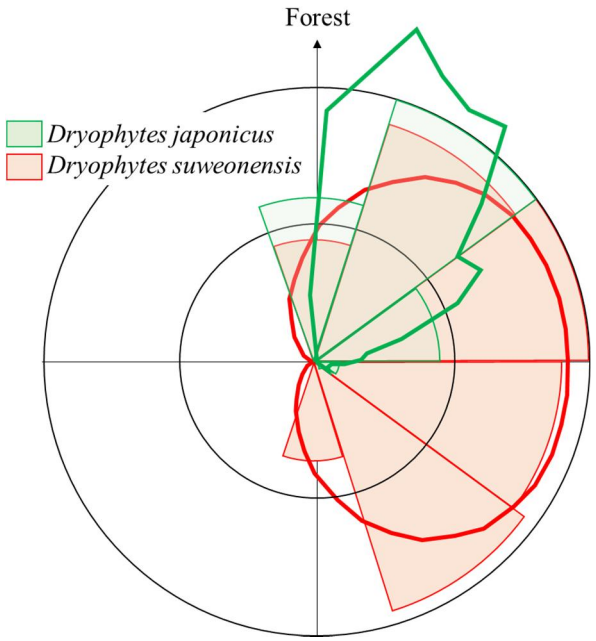


Figure 8.5. Orientation of the path taken *Dryophytes japonicus* individuals (green) and *D. suweonensis* individuals (red) during the fall tracking experiment, when corrected for release point for each individual. *Dryophytes japonicus* individuals were directed towards forests in 66.2 % of cases and *D. suweonensis* individuals moved towards rice paddies in 41.14 % of cases and towards forests in 16.4 % of cases. The dark line is a kernel density estimate representative of the weighted relative directionality to forest and the light shaded areas are abundances proportional to radius.

Once tested statistically, the logistic regression model was not significant, $\chi^2_{(1)} = 0.56$, $p = 0.453$, explaining 0.1 % (Nagelkerke pseudo-R^2) of the variance in directionality, correctly classified 55.4 % of cases. There was thus a difference between the two species, with *D. japonicus* displaying a significant preferential direction, while *D. suweonensis* did not.

The results of the GLM explaining the factors related to directionality (Table 8.2) show that only the distance travelled was significant. Despite the different distances to forest at the four sites (139, 1018, 470 and 404 m), no significant variations between sites were reported, and it was the same for the variation between individuals, highlighting a general behaviour (Table 8.2). The average distance moved towards the rice paddies between two location check was 78.75 ± 235.98 cm while the distance travelled towards the forest was on average - 38.18 ± 219.34 (Fig. 8.4). When assessing the difference in directionality through angles of displacement for the two species, we found a difference in directionality as the angle described by the movements of *D. japonicus* deviated from the forest by 34.79 ± 5.09 (mean ± kappa) degrees only on average, while the angle described by *D. suweonensis* was 87.55 ± 1.79 degrees away from the forest on average (Fig. 8.5). The difference between the two species was found to be significantly different (Mardia-Watson-Wheeler test; $W = 461.80$, $n = 288$, $p < 0.001$).

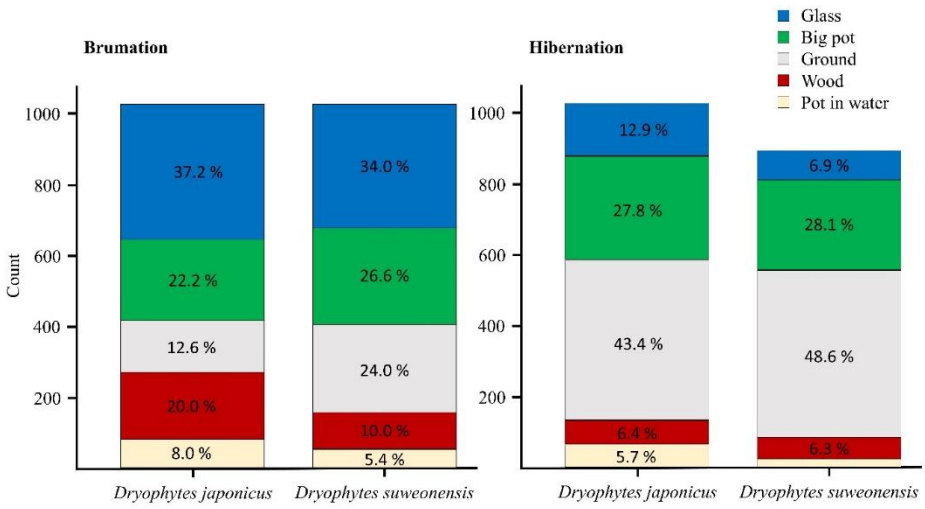


Figure 8.6. Microhabitat variation displayed by *Dryophytes suweonensis* and *D. japonicus* during brumation and brumation with corresponding frequency. The variation in the total number of counts come from the death of three individual *D. suweonensis* during the period before hibernations. The non-annotated stack "pot in water" for *D. suweonensis* is 1.7 %.

Table 8.2. GLM to test the relation between directionality and other factors collected during the tracking experiment to investigate the brumation behaviour of *Dryophytes suweonensis.*

		Type III - $\Sigma\chi^2$	*df*	χ^2	*F*	*P*-value
Distance travelled	Hypothesis	115.53	75	1.54	9.53	< 0.001
	Error	34.42	213	0.16		
Site	Hypothesis	0.39	2	0.19	1.19	0.306
	Error	34.42	213	0.16		
Sex	Hypothesis	0.02	1	0.02	0.15	0.700
	Error	34.42	213	0.16		
Vegetation	Hypothesis	0.61	3	0.20	1.27	0.286
	Error	34.42	213	0.16		
Date	Hypothesis	0.29	1	0.29	1.80	0.181
	Error	34.42	213	0.16		
Time	Hypothesis	0.21	1	0.21	1.29	0.258
	Error	34.42	213	0.16		
Temperature	Hypothesis	0.18	1	0.18	1.14	0.286
	Error	34.42	213	0.16		
Height	Hypothesis	0.02	1	0.02	0.10	0.750
	Error	34.42	213	0.16		
Lux	Hypothesis	0.03	1	0.03	0.18	0.668
	Error	34.42	213	0.16		
Individual ID	Hypothesis	0.05	1	0.05	0.33	0.568
	Error	34.42	213	0.16		

***(3) L**aboratory brumation and hibernation observations*

Brumation

During the brumation period, a significant difference in microhabitat use was observed between the two species (Table 8.3), under a significant model ($\chi^2 = 3266$, $df = 212$, $p < 0.001$) and explaining 85.9 % or variation (Nagelkerke pseudo-R^2). *Dryophytes japonicus* had a preference for the wood microhabitat (20.0 % use in *D. japonicus* and 10.0 % in *D. suweonensis*) and *D. suweonensis* had a preference for the ground microhabitat (24.0 % use in *D. suweonensis* and 12.6 % use in *D. japonicus*; Fig. 8.6). These microhabitats were the most commonly used ones after removing the "glass" microhabitat used for displacements (37 % in *D. japonicus* and 34.0 % in *D. suweonensis*).

One of the other significant differences between the two species was the height at which they were found in the terrariums (Table 8.3). *Dryophytes japonicus* was on average 23.14 ± 4.33 cm high, while *D. suweonensis* was on average 20.37 ± 7.37 cm high (Fig. 8.7). Besides, there was a significant difference between individuals and families. The model for this analysis was significant ($\chi^2 = 373.48$, $F = 264.41$, p-value < 0.001).

Hibernation

During the hibernation period, the variables microhabitat and height were also significantly different between the two species (Table8.

4), under a significant model (χ^2 = 2138.21, *df* = 232, $p < 0.001$) and explaining 79.1 % or variation (Nagelkerke pseudo-R^2). In this second phase of the experiment, the use of the flooded microhabitat was higher for *D. japonicus* (5.7 %) than for *D. suweonensis* (1.7 %) while *D. japonicus* moved about twice more than *D. suweonensis*, as seen by a larger use of windows (12.9 versus 6.9 %; Fig. 8.6).

According to the results of the brumation experiment, the height at which individuals were found was also significantly different (Table 8.4), with here as well *D. japonicus* on average higher (7.19 ± 9.47 cm) than *D. suweonensis* (5.21 ± 8.10 cm; Fig. 8.6). Besides, there was a significant difference between families. The model for this analysis was significant (χ^2 = 463.33, *F* = 663.08, *p*-value < 0.001).

Difference between brumation and hibernation:

The Multinomial Logistic Regression to assess whether the two species differed in microhabitat use between brumation and hibernation were significant for species (χ^2 = 44.83, *df* = 4, $p < 0.001$), phase (χ^2 = 118.27, *df* = 4, $p < 0.001$) and height (χ^2 = 4362.12, *df* = 4, $p < 0.001$). The model was significant (χ^2 = 5069.81, *df* = 12, p <0.001) and explanatory for 78.9 % of the variance (Nagelkerke preudo-R^2). As seen earlier, the frequency of use for the wood microhabitat decreased between the two species between brumation and hibernation, while the frequency increased for the ground microhabitat. The average height also decreased for the two

species at the same period (Fig. 8.6).

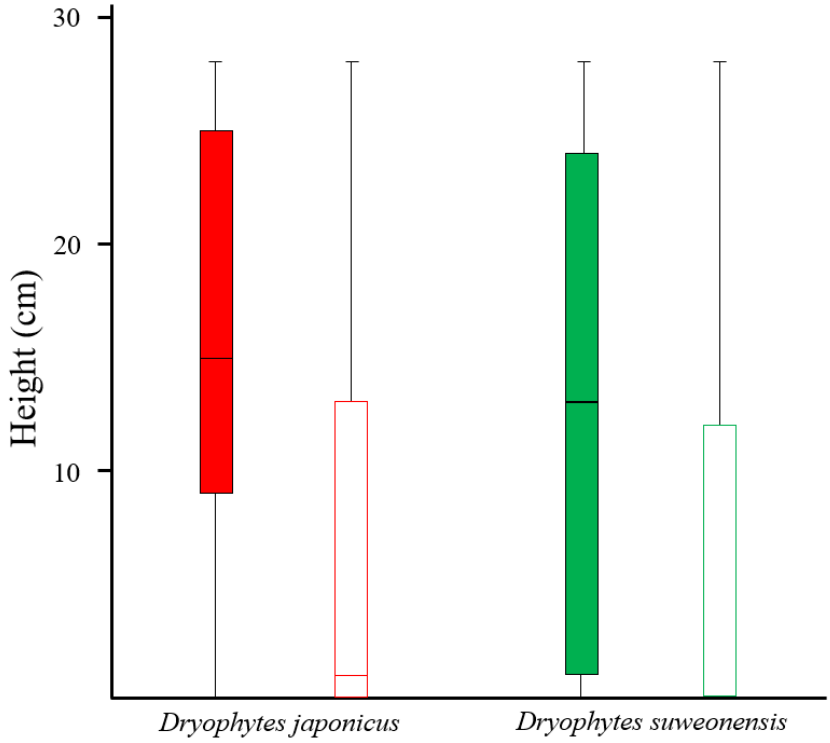


Figure 8.7. Height difference between *Dryophytes japonicus* and *D. suweonensis* during the brumation (solid) and hibernation phases (hollow) of the lab experimentation.

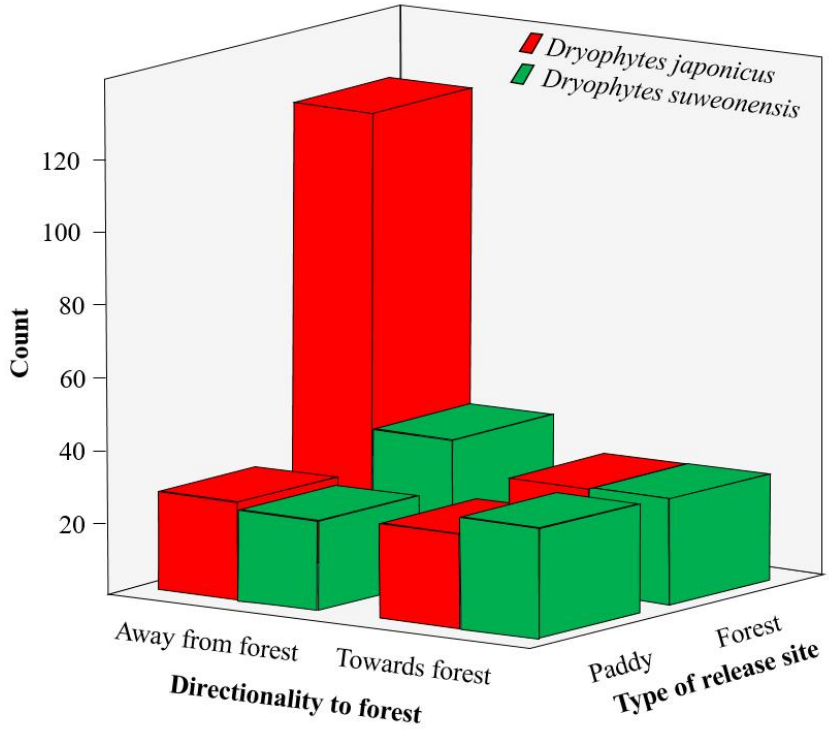


Figure 8.8. Figure representative of the difference in the directionality of displacements shown by *Dryophytes suweonensis* and *D. japonicus* in relation to the type of sites where they were released and the directionality to forests.

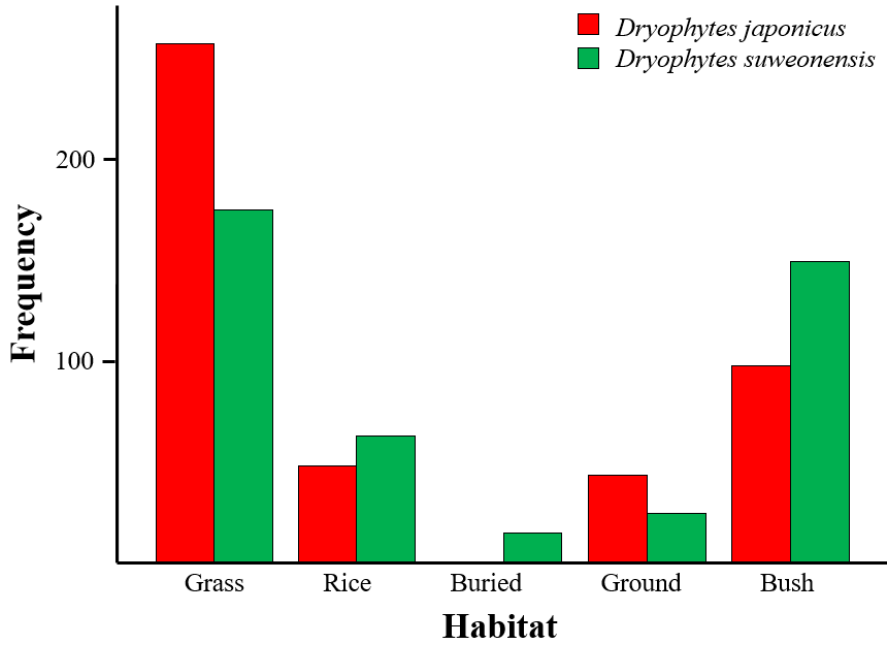


Figure 8.9. Figure representative of the variations in habitat use between *Dryophytes suweonensis* and *D. japonicus* during the spring tracking experiments.

Table 8.3. Results of the Multinomial Logistic Regression ran to revel variations in microhabitat use between the two species during the brumation experiment (n = 2055).

	χ^2	*df*	*P*-value
Species	30.53	16	< 0.001
Family	59.45	16	< 0.001
ID(Family)	514.65	176	< 0.001
Temperature	13.42	4	0.009
Height	2260.70	4	< 0.001
Time	5.62	4	0.229
Date	44.35	4	< 0.001

(4) Winter field

Out of the four hours spent digging at each of the sites, a single individual was found. It was a female *D. suweonensis* at the paddy site in Sihung (site 1; 37.410046° N; 126.808053° E; Fig. 8.2). The individual was found within a burrow excavated by another animal, absent during the finding. The individual was buried between 27 and 30 cm deep. The absence of other findings does not reflect the absence of individuals, but out inability to find them.

(5) S*pring orientation tracking*

The directionality exhibited by *D. japonicus* and *D. suweonensis* during the spring tracking experiment was significantly different between the two species (Table 8.5). When released in the forest habitat *D. japonicus* moved 60.1 % away from the forested release point but also 42.6 % towards paddies (Table 8.6), a significant difference between the two directions for the species (T-test; t = - 8.15, df = 127, $p < 0.001$). On the other hand, there was no difference in directionality between the two species when released in the rice paddies (Table 8.6; T-test; t = - 1.31, df = 45, p = 0.198). Oppositely, *D. suweonensis* movements towards and away from the forest when released in that habitat was no significantly different (Table 8.6; T-test; t = - 3.49, df = 50, p = 0.186), neither than it was when released in rice paddies (Table 8.6; T-test; t = - 0.32, df = 46, p = 0.749; Fig. 8.8). This pattern was the same for the cumulated distance travelled

by the two species towards either rice paddies (*D. japonicus* = 20.60 ± 128.62 and *D. suweonensis* = 15.82 ± 119.01 m; ANOVA; $\chi^2 = 402.09$, $F_{1,872} = 0.07$, $p = 0.786$) or forests (*D. japonicus* = - 30.46 ± 119.36 and *D. suweonensis* 0.98 ± 115.80; ANOVA; $\chi^2 = 31439.84$, $F_{1,872} = 6.61$, $p = 0.010$; here cumulated by type of site for ease of understanding).

The results of the first GLM also show a difference in microhabitat use between the two species (Table 8.5), with grass preferentially used by *D. japonicus*, and grass and bush principally used by *D. suweonensis*, while *D. japonicus* was never found buried and *D. suweonensis* was found buried in only 3.3 % of cases, and on the bare ground only 6.3 % of cases (Fig. 8.9).

When assessing the difference in movement angles for the two species (Fig. 8.10), the angles described by the two species when released in rice paddies: 79.22 ± 1.69 (mean ± kappa) for *D. japonicus* and 68.02 ± 2.00 for *D. suweonensis,* and forests: 113.77 ± 1.87 for *D. japonicus* and 86.39 ± 1.57 for *D. suweonensis,* were significantly different (Mardia-Watson-Wheeler tests) for: habitat of release for both *D. japonicus* ($W = 364.76$, $n = 225$, $p < 0.001$) and *D. suweonensis* ($W = 326.79$, $n = 221$, $p < 0.001$) but also between both species for a given release habitat: rice paddies ($W = 306.23$, $n = 192$, $p < 0.001$) and forests ($W = 387.23$, $n = 245$, $p < 0.001$).

Table 8.4. Results of the Multinomial Logistic Regression ran to revel variations in microhabitat use between the two species during the hibernation experiment (n = 1750).

	χ^2	df	P-value
Species	34.06	4	< 0.001
Family	72.05	16	< 0.001
ID(Family)	258.58	196	0.002
Temperature	16.80	4	0.110
Height	1738.50	4	< 0.001
Time	2.72	4	0.606
Date	3.51	4	0.045

DISCUSSION

Extensively different traits can occur with little genetic change (West-Eberhard, 2005), and the suspected divergence in evolutionary history between the two Korean *Dryophytes* species (Kim, 2016) is here clearly confirmed. While *D. japonicus* migrates twice yearly between rice paddies and forested areas, to breed and over-winter, *D. suweonensis* is present at rice paddies all year round, and hibernates buried underground. The ancestors of the two species diverged during the Late Miocene (~6.5 Mya; Dufresnes et al, 2016), supposedly when one of the two species adapted to a different environment. It is likely that this happened when *D. suweonensis* preferentially selected marshes for breeding, while the preference of the ancestral species is expected to have been broader. Breeding in a different habitat led to the acquisition of new traits, such as holding on vegetation while calling (Borzée et al, 2016b), but also relying on the same habitat for hibernation, and thus losing the ancestral character: migration. It is unlikely that the seasonal migration is a newly acquired trait in *D. japonicus* as the species breeds in most types of habitats, also at higher elevations (Roh et al, 2014), and thus migration is expected to have been the ancestral character. The length of the migration distance may have however been modified by the development of agriculture, with the two species brought back into contact (Borzée et al, 2017; Borzée et al, 2015b).

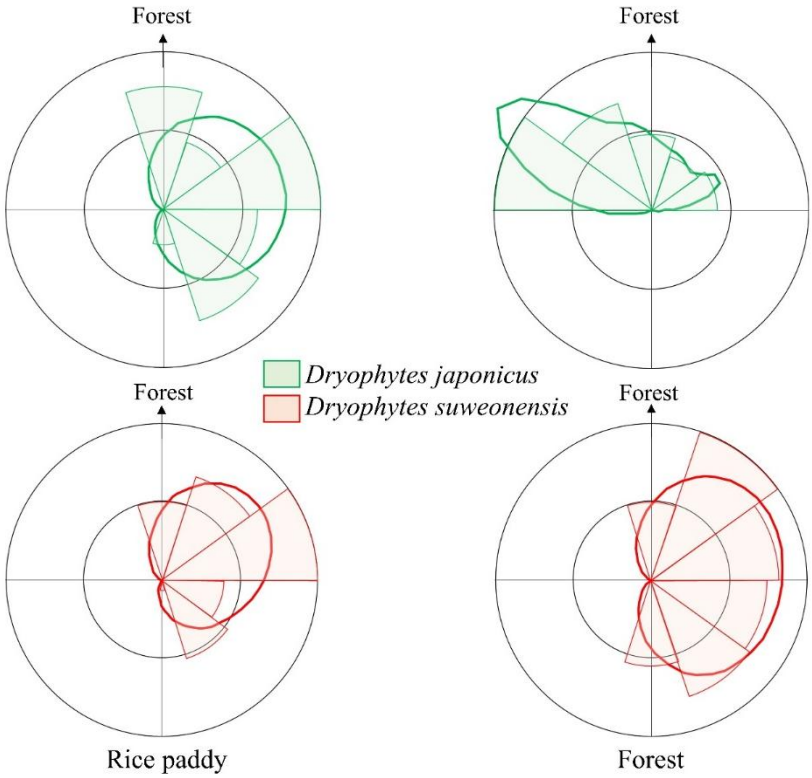


Figure 8.10. Rose diagrams representative of the variation in the angle of displacement between *Dryophytes japonicus* and *D. suweonensis* in relation to the release habitat (rice paddies or forest). The difference in angle of displacement is significantly different between the two types of environment for *D. japonicus* for both species. The dark line is a kernel density estimate representative of the weighted relative directionality to forest and the light shaded areas are abundances proportional to radius.

Table 8.5. Results of the GLM to differentiate the factors of importance between the two species during the spring tracking experiment.

	Type III - $\Sigma\chi^2$	*df*	χ^2	*F*	*P*-value
Species	1.14	1	1.14	4.25	0.040
ID	7.22	24	0.30	1.12	0.309
Habitat	5.66	4	1.41	5.29	0.000
Date	0.28	1	0.28	1.06	0.304
Time	0.11	1	0.11	0.40	0.527
Temp	0.07	1	0.07	0.26	0.610
Height	0.04	1	0.04	0.16	0.686
Error	224.48	839	0.27		

Table 8.6. Descriptive statistics for the directionality of the movements exhibited by *Dryophytes suweonensis* and *D. japonicus* in relation to the type of site selected for the release (i.e. rice paddy or forest), during the spring tracking experiments. Data pooled two-by-two do not reach 100 % as frogs were sometime immobile and thus not providing any directionality data for a few hours.

Habitat	Species	
Forest	*D. japonicus*	*D. suweonensis*
Directionality in relation to forest		
Away from forest	60.1	56.7
Towards forest	13.9	43.3
Directionality in relation to paddy		
Away from paddy	15.9	22.4
Towards paddy	46.2	77.6
Paddy		
Directionality in relation to forest		
Away from forest	50.9	45.5
Towards forest	49.1	54.5
Directionality in relation to paddy		
Away from paddy	40.4	40.0
Towards paddy	59.6	60.0

Dryophytes suweonensis breeding in a separate habitat is the preferential hypothesis as this scenario enables the development of pre-zygotic isolating traits, such as seen in Dryophytes cinereus (Höbel et al, 2003), and the two species are able to hybridise (Kuramoto, 1984; Borzée et al, 2015b), and have thus not evolved post-zygomatic isolation. Sympatric speciation is hypothesised as peri- and para-patric speciation cannot have occurred with the two species displaying sympatric ranges (Jang et al, 2011).

During the breeding season, both species are calling from rice paddies, and while calling activity is discontinued in early July (Roh et al, 2014), both species are present in the vicinity of rice paddies until mid-September, where individual sometimes produce calls, although unlikely for mating purposes, with juveniles attempting the same behaviour in synchrony (Pers. Obs.). At this point in time, adult *D. japonicus* migrate towards forests, up to several hundred meters away in this study, although closely related species can migrate up to 8 km (Angelone and Holderegger, 2009). Males *D. japonicus* will be present on the tree canopy, favouring oak trees (Borzée et al, *in review-a*), and producing calls, as observed during transects in this study, until they are not seen anymore, in late Ocober/Early November, temporarily matching with the first frost. Adult and juvenile *D. suweonensis* are staying in the vicinity of rice paddies, favouring the upper leaves of planted beans, hypothetically

for feeding based on the very high insect density seen in proximity. This is however also making the species susceptible to the bean harvest, potentially impacting the recruitment of young individuals into the breeding pool. Individuals will then find shelter underground on the banks of rice paddies, where rice straw is stacked after harvest, but also burnt before the thaw of ice, with unknown consequences on the species.

Both before and after overwintering, the two species displayed variations in the orientation of their displacements in relation with forests and rice paddies. *Dryophytes japonicus* was clearly aiming at forests before winter, while *D. suweonensis* displayed non-directional displacements. After winter, the difference between the species was still present. *Dryophytes japonicus* was clearly aimed at the breeding sites when released from the forest, while *D. suweonensis* was still not displaying any directionality, and would have been qualified as lost if from a species with higher cognition level. When released in rice paddies, both of the species were displaying non-directional displacements, an indication that they had reached their breeding sites, and that the directionality displaying by *D. japonicus* towards forests before hibernation and towards rice paddies after hibernation was not an artefact. This raise an interesting question on the methods used to display directionality. Amphibian species are known to rely on a set of methods to orientate their movement (see review by Sinsch, 1990). However, as all

individuals had been kept in laboratory for a month prior to release, or were laboratory born, landscape and field recognition could not be learned. Also, rainy conditions during the fall orientation experiment disqualify star recognition and the use of polarised light.

The behaviour expressed by the two species while kept in terrariums during fall and winter was clearly different between species and between seasons, according to what would have been expected from wild individuals. During brumation, *D. suweonensis* was preferentially found on the ground of the terrarium, while *D. japonicus* was more often found on wooden structures, at higher heights from the ground. This matches the brumating behaviour recorded in the wild, with *D. suweonensis* brumating in the vicinity of rice paddies, and thus in an area without high vertical structures, while *D. japonicus* is found on trees most of the time. The preference for bean leaves by *D. suweonensis* in the wild, circa 50 cm high, may thus be more closely related to prey availability than microhabitat preference. The hibernation period saw the two species exploiting the ground microhabitat preferentially, and thus hints at the use of buried hibernaculum by the two species to hibernate. *Dryophytes japonicus* was more active and found higher during the period, in agreement with the high freeze tolerance shown by the species (Storey and Storey, 2017).

Amphibians have been the subject of strong population declines

over the last decades (Blaustein et al. 1994), with currently approximatively a third of all species under threat of extinction, and more than two hundred already extinct (Stuart et al. 2004). The potential for extinctions in pristine environments, such as Madagascar (Kolby, 2014) is still very high and basic ecological knowledge is still required for a high number of species, such as highlighted by the "data deficient" species of the IUCN Red List of endangered species (IUCN 2014). In response to the loss, biodiversity conservation efforts have been deployed to tackle population decreases and extinctions (Marsh 2008, Gascon 2007). However, and especially with amphibians, most conservation work is addressed to the breeding habitat, and thus, not all conservation efforts have been successful (Blaustein & Kiesecker 2002). Failure is not necessarily due to the work carried, but because of limiting factors such as basic knowledge. We therefore urge the implementation of conservation measures for *D. suweonensis*, listed as endangered under the IUCN (2017) and the Korean law (Ministry of Environment, 2012), highlighting that protecting the totality of the space used by the species is easier than for most species, as hibernation and breeding habitats overlap. Despites these being only in rice paddies, international conservation plans such as the ones developed by RAMSAR allow for this dual objective.

Appendix four

INTERFERENCE COMPETITION DRIVEN BY HYDRIC STRESS IN KOREAN HYLIDS.

ABSTRACT

Competition is based on the type and importance of the resource sought, and interactions intensify with the importance of a given resource. One of the highest degree of interference competition is physical interactions for a given resource. The treefrogs *Dryophytes japonicus* and *D. suweonensis* compete for spatial resources for the production of advertisement calls. Here, we investigate a case of direct interference competition due to artificial hydric stress caused by variations in the irrigation system of rice paddies. Under this specific condition, an unusually high number of males of both species congregated in a single flooded field. We first found that males segregated following a now demonstrated pattern, with *D. japonicus* on the edges and *D. suweonensis* at the centre of the flooded area. However, the distance-to-bank for amplexuses of the two species were not significantly different. Due to the high number of individuals and the small size of the site, the area used by males both species partly overlapped, and thus lead to a physical interactions between two males of the different species. *Dryophytes japonicus* took over a calling site and managed to remove the opponent

male. We sequentially described the interaction here, potentially representative of the general outcome of interference competition between these two species.

INTRODUCTION

All organisms need resources and more often than not these are in short supply, thus resulting in competition (Schluter, 2000). Although attenuated by specialisation and niche selection (Turelli et al., 2001), over time, competition may lead to divergences (Lack, 1947; Schluter, 2000) and possibly speciation (see Darwin's finches example; Grant and Grant, 2002). Competition can be further dichotomised between exploitative and interference competition. For instance, intraspecific interference competition in *Rana utricularia* tadpoles supplanted exploitative competition (Steinwascher, 1978), and intraspecific visual interference competition played a role in larval development in *R. kukunoris* (Yu and Lambert, 2015). However, the two types of competition are non-mutually exclusive, see for instance competition between *Ambystoma talpoideum* and *A. maculatum* (Walls and Jaeger, 1987) and between *R. temporaria* and *Bufo calamita* (Griffiths et al., 1991).

Interference competition occurs between species both in direct and indirect competition. For instance, the larvae of the salamander *Ambystoma maculatum* and *A. talpoideum* directly compete over hides

(Walls, 1990). An examples of indirect interference competition is the production of growth inhibition chemicals by the tadpoles of *B. calamita* and *R. temporaria* (Beebee, 1991). However, because of species-specific abilities to exploit resources, the impact of competition can be either symmetric or asymmetric (Begon et al., 2006). For instance, when in sympatry with *R. blairi*, the length of the larval period will increase and the metamorphic body mass will decrease in *R. areolate*, while *R. blairi* will experience a drop in the number of metamorphosing individuals (Parris and Semlitsch, 1998).

On the Korean Peninsula, *Dryophytes japonicus* and *D. suweonensis* (previously *Hyla*; Duellman et al., 2016), are competing with each other for calling space in rice paddies. The calling space used by *D. suweonensis* is limited by the calling activity of *D. japonicus*, but not vice-versa (Kim, 2015; Borzée et al., 2016a; Borzée et al., 2016b). Here, we report the first case of interference competition between these two treefrog species. Physical encounter between calling males of the two species does not usually occurs due to habitat segregation (Borzée et al., 2016a), following historical differential use of the environment (Kim, 2016; Borzée et al., 2017). However, in this special case of human-induced hydric stress, the two species were brought in physical contact.

MATERIAL AND METHODS

This abnormal situation of hydric stress during the peak breeding season was observed on 27 June 2015 at 20:25 pm in Chungju, Judeok-up, Jaenae-ri 618 (36.989295°N; 127.782439°E). Because of the lack of rain, most rice paddies had been drained of agricultural water, while a lone fallow field was flooded by a ruptured farming water hose (Fig. 1). The site was similar to the only natural site reported for *D. suweonensis* in term of spatial setting, but different by its vegetation (Borzée and Jang, 2015). This attracted an abnormally high number of males and females from both treefrog species.

Spatial position of frogs and physical confrontation

For each calling frog within the submerged field, we recorded its position in relation to the bank with a range finder (SD 60; Sincon; Taichung, China), at 5 cm resolution. Data were collected for 10 male *D. japonicus*, two amplexed *D. japonicus* pairs, 16 male *D. suweonensis* and three amplexed *D. suweonensis* pairs. While measuring the distance to bank for all calling individuals, we detected a male *D. japonicus* moving towards a calling *D. suweonensis*. We described sequence by sequence the resulting physical struggle between the two individuals.

Statistical analysis

To confirm the observation by Borzée et al. (2016b) on

microhabitat segregation, we ran a Mann-Whitney U test on the distance to bank between the two species. Then, to assess the continuity of the pattern, we ran a Mann-Whitney U test between the distances to bank for the amplexuses of the two species. Mann-Whitney U tests were selected due to the non-normal distribution of the data, but the similar shape of each dataset. All statistical analysis were run with SPSS v21.0 (SPSS, Inc., Chicago, IL, USA), with *p*-values set at 0.05 for significance. The physical interaction between the males of the two species is described in the form of an ethogram.

RESULTS

The site was trapezoidal, with the two parallel edges 4.40 and 14.10 m long, and the longest edge 52.35 m long (Fig. A4.1). The water was between 5 and 60 cm deep, with a modal depth of 40 cm.

Spatial positioning

Male *Dryophytes japonicus* (n = 10) were on average 1.79 ± 0.83 m (mean ± SD) away from the closest bank (range = 0.53 - 3.53 m) and male *D. suweonensis* (n = 16) were on average 4.48 ± 1.09 m away from the closest bank (range = 2.98 - 6.10 m). This is a significant difference between the positions of the two species (Mann-Whitney U test; $U = 5.00$, $Z = -3.95$, $p < 0.001$).

The amplexed *D. japonicus* pairs (n = 2) were on average 3.77 ± 0.79 m away from the closest bank, while the amplexed *D. suweonensis* pairs (n = 3) were on average 4.78 ± 0.51 m away from the closest bank. The distance to bank for the amplexus of the two species was not significantly different (Mann-Whitney U test; U = 1.00, Z = - 1.15, p = 0.248).

Physical interaction

The physical interaction between the males of the two species took place 3.05 m away from the bank, 12.45 m away from the longest of the two parallel banks (Fig. 1). This was within the overlap between the two species described above (2.98 to 3.53 m away from the closest bank).

The male *D. suweonensis* was calling on a wooden twig, roughly parallel to the water, 6.2 cm above water level (Table A4.1). The male *D. japonicus* jumped from the bank, swam towards the twig, and set to climb on it. Once within reach, the male *D. japonicus* attacked the male *D. suweonensis*, which had stopped calling when the other male reached the twig. The male *D. japonicus* aimed at biting while "pushing/taking support" on the body of its opponent with its forelimbs. The attack stopped two sec later, with the male *D. japonicus* taking two steps backward, and the two opponents staying immobile for four more seconds. The male *D. japonicus* then walked towards the male *D. suweonensis*, which took three steps backwards and jumped to the water.

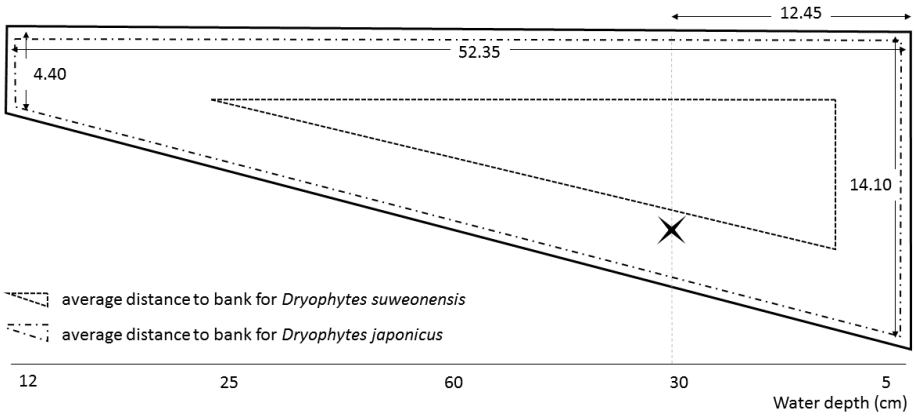


Figure A4.1. Line drawing of the site where the physical interaction between the two individuals took place. The inner-dotted-line is the average distance to bank for *Dryophytes suweonensis* (4.48 m) and the outer-dotted-line is the average distance to bank for *D. japonicus* (1.79 m). The star represents the place where the interaction between the two species took place.

Table A4.1. Descriptive ethogram for the physical confrontation between the male *Dryophytes japonicus* and the male *D. suweonensis*. The calling activity displayed by either male before and after the confrontation is representative of the purpose of the fight: the acquisition of a calling perch.

Time (sec)	Male *Dryophytes suweonensis*	Male *Dryophytes japonicus*
-	Calling	Swimming
-	Non-calling	Climbing on twig
0	Immobile	Biting
2	Immobile	Withdrawing
6	Immobile	Immobile
7	Jumping away	Moving forwards
18	-	Calling

The total length of the even lasted seven sec, from first physical contact to the spontaneous self-withdrawal of the male *D. suweonensis* jumping into the water (Table A4.1). The male *D. suweonensis* escaped towards the centre of the water body and the male *D. japonicus* started producing advertisement calls 18 sec later.

DISCUSSION

Our observations report a second type of competition between *Dryophytes japonicus* and *D. suweonensis*: interference competition for calling sites, in non-natural conditions. At the difference with other studies (Borzée et al., 2016b; Kim, 2015), there was an overlap between the maximum distance to the bank for *D. japonicus* and the minimum distance to the bank for *D. suweonensis*. This overlap likely results from the hydric stress bringing all frogs together in an area where a ruptured water pipe flooded a fallow rice paddy. As the physical interaction took place within this area, we hypothesise that it was an artefact resulting from the human modifications to the landscape. This is also the highest number of *D. suweonensis* reported at a single calling site, and thus resulting in interference competition for a rarefied resource. We expect the size of the site not to be the key to the interpretation of these results, as segregation at smaller sites has been observed (Borzée et al., 2016b). However, the object of the competition, *i.e.* a calling perch, is readily understandable.

The male *D. suweonensis* escaping towards the centre of the water body, usually not populated by *D. japonicus*, follows the ecological knowledge on microhabitat use for these species. It is however interesting to note that although non-significant, the distance to the bank for the amplexuses of the two species follows the same segregation pattern. It is so far unclear if the low sample size is the reason for the non-significance in distance to bank between the two species, or if females move around to lay eggs in a random fashion to increase offspring survivorship, such as for Bibron's toadlet (*Pseudophryne bibronii*; Byrne and Keogh, 2009).

The outcome of the physical interaction may be the result of morphological differences between the two species as males *D. japonicus* are on average 5.22 % larger than males *D. suweonensis* for snout-vent length (Borzée et al., 2013). Another explanation is that despite male-male physical confrontations being common in *D. japonicus*, this is the first such observation for *D. suweonensis* and the species may not express this behaviour. Besides, the lack of behavioural answer from *D. suweonensis* to the attack by *D. japonicus* could be interpreted as the result of signals non-perceivable by the observer, such as chemical communication observed in reed frog species (Hyperoliidae; Starnberger et al., 2013). Our observations also potentially explain the dynamics for the numerous *D. japonicus* and the endangered *D. suweonensis* in Korea. When calling sites are limited, interference competition could be a contributing factor to

the decline of *D. suweonensis*.

Appendix five

MATING BEHAVIOUR OF FEMALES *DRYOPHYTES SUWEONENSIS*.

Female mating behaviour and mate choice in anurans are among the leading factors of sexual selection (Ryan 1980). Gravid females express their preferences based on male advertisement signals, including acoustic, visual, chemical and physical cues, before directing their displacement towards the male of interest. In Hylidae, this behaviour is typically expressed through phonotaxis (Gerhardt 1991). Most studies on female mating behaviours have focused on preferences in experimental arenas and are thus isolated from natural settings.

Dryophytes suweonensis is an endangered treefrog species from the Korean peninsula. It almost exclusively breeds in rice paddies, due to the loss of natural breeding sites following wetland reclamation (Borzée and Jang 2015). During the breeding season, male *D. suweonensis* produce advertisement calls starting mid-afternoon, taking advantage of rice seedlings as calling perches (Borzée et al. 2016a). In contrast, the abundant *D. japonicus* generally calls after sunset from the edge of water bodies. The temporal call segregation acts as a pre-mating isolation mechanism decreasing potential hybridization between the two species.

Females mating behaviour was recorded during the breeding seasons of 2015, 2016 and 2017. Three females were found holding onto rice seedlings alone, between 10 cm and 25 cm above the water level, presumptively looking for males (at 2240 h on 17 June 2015 at 36.8789°N, 126.9633°E, at 2020 h on 24 May 2016 at 35.9359°N, 126.9943°E (Location 1), at 1645 h on 29 May 2017 at 36.252238°N, 126.847122°E (Location 2), WGS84). Six additional females were observed climbing up rice seedlings to approach calling males: one individual at 2127 h on 24 June 2015 at 35.991°N, 126.928°E, three individuals between 1700 h and 2345 h on 26 May, 29 May and 4 June 2017 at Location 2, one individual at 1920 h 2017 at location 1 and a last individual at 0050 on 5 June 2017 at 37.643646°N, 126.421282°E (WGS84; Fig. A5.1a). In addition, eight amplectant pairs were observed holding onto rice seedlings between 3 and 15 cm above water, between 2130 h and 0000 h (24 June 2015, 35.991°N, 126.928°E; 24 June 2015, 37.7960°N, 126.8107°E; 26 June 2015, 35.980°N, 126.936°E; 27 June 2015, 35.976°N, 126.894°E; and 29 June 2015, 36.9896°N, 127.7747°E, 29 May and 4 June 2017 at Location 2, WGS84; Fig. A5.1).

For all six females observed climbing on rice seedling, males were recorded to be continuously producing advertisement calls for the whole duration of the climb, thus inducing phonotaxis, by female individuals and human observers. All amplectant pairs and lone females

hanging from rice seedlings were found through random search patterns, without cues to enable their localization by researchers. Although males were recorded calling until 0330 h, no female was observed after 0050, and only seemingly smaller males were observed calling later at night.

Positive phonotaxis was expected as most female hylidae approach males to induce the first contact, prior to successful mating. Rice fields may provide adequate substitute breeding sites for *D. suweonensis*, similar to their natural breeding habitat (Borzée and Jang 2015) and it is thus impossible to classify the female mating behaviour as natural or resulting from the anthropologically modified environments. Our data highlights the importance of understanding microhabitat use by this species in order to comprehend species specific breeding behaviours in the frame of species conservation and restoration.

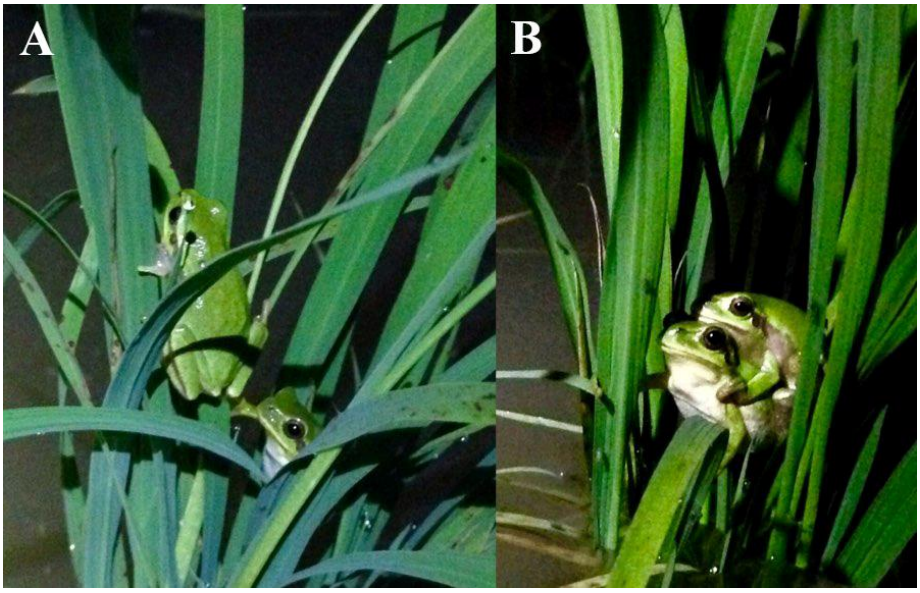


Figure A5.1. (A) Female *D. suweonensis* (bottom) approaching a calling male (top) and (B) an amplectant pair of *D. suweonensis* on rice seedlings.

Appendix six

MICROHABITAT USE DURING BRUMATION IN THE JAPANESE TREEFROG, *DRYOPHYTES JAPONICUS.*

ABSTRACT

Although amphibians undergo drastic changes in physiology and behaviour before hibernation, this phase of their life cycle (i.e., brumation) is the least understood. We investigated the patterns of microhabitat use by *Dryophytes japonicus* during brumation using a Harmonic Direction Finder to track 27 adults in October 2013. Most frogs used chestnut trees throughout their diel cycle. The species was most active within the "leafy vegetation" microhabitat, moving about 2 m within 72 h on average, and mostly *circa* 10 AM. Frogs moved less in the four other microhabitats, with individuals moving between 1 m and 50 cm and typically occurring in the early afternoon. Around 3 pm, the microhabitat mostly used was "on bark", with displacements almost totally halted. The use of microhabitats and shelters, as well as movements in relation to time of day, suggests that *D. japonicus* displays behavioural thermoregulation during brumation. This research is the first providing insights in the brumation ecology of a non-freeze-resistant Palearctic anuran.

INTRODUCTION

Despite very early research on (probably) *Rana sylvatica* (Swaine et al, 1798, Tyrrell, 1911), the natural history of over-wintering Palearctic anurans is very little studied. For instance, the overwintering biology of European Hylids is still so far best characterized by opportunistic notes (reviewed by Stumpel, 1990). Data from North America are a decade more recent, and describes Wood Frogs (*R. sylvatica*) as occupying upland forest habitat during late fall and winter (Regosin et al, 2003). Anurans overwintering underwater are better studied, focusing for instance on the Northern Leopard Frog (*Rana pipiens*; Cunjak, 1986), the Mountain Yellow-legged Frog (*R. muscosa*; Bradford, 1983) or the American Bullfrog (*Lithobates catesbeianus*; Stinner et al, 1994).

In Hylidae, in broad taxonomic and behavioural terms (Moen et al, 2013), freeze tolerance independently evolved once or twice (Voituron et al, 2009). This physiological property is observed for instance in *Hyla versicolor* and *Pseudacris crucifer* (reviewed by Storey and Storey, 1992), *H. chrysoscelis* (Costanzo et al, 1992), *H. regilla* (Croes et al, 2000) or *P. triserata* (Edwards et al, 2000) in North America; *H. arborea* in Europe (Voituron et al, 2009) and *Litoria ewingi* in Oceania (Bazin et al, 2007).

Palearctic treefrogs are partially active during the pre-hibernation period, called brumation for ectotherms (Mayhew, 1968, Pratihar and

Kundu, 2011, McEachern et al, 2015). The brumation period usually last until November (Stumpel and Hanekamp, 1986, Stumpel, 1990, Sugimoto and Jiang, 2008), at which point treefrogs shelter to survive to the coldest temperature through winter torpor. Re-emergence typically occurs between March and June (Harris, 1975, Stumpel, 1990, Sugimoto and Jiang, 2008). However, awakening periods may take place on an opportunistic basis, and temperatures above seasonal averages may result in short term emergence, such as seen for *H. arborea* in December (Beebee, 1988) and on 24 December 2013 (Author Pers. Obs. T = 12.4 °C, rH = 66 %, Béraut, France).

Sheltering in specific microhabitats limits contact with the cold (Schmid, 1982, Stumpel, 1990), and overwintering sites are typically above average temperatures (Van Gelder et al, 1986). Shelters can be under water, buffering against extreme cold, (e.g. *H. arborea,* Stumpel, 1990; presently *H. orientalis*, Stöck et al, 2012). This type of overwintering has the advantage of providing rapid access to the breeding site after brumation, but also presents the drawback of possible anoxia or hypoxia (*e.g. Acris crepitans*, Irwin et al, 1999). On the other hand, terrestrial hibernacula are linked to an increased risk of freezing, although individuals overwintering in terrestrial hibernacula do not require waiting for the ice to thaw to emerge. Thus, terrestrial hibernacula provide an early spawning advantage, which may lead a better reproductive success

(Stumpel 1990). A literature survey conducted here indicates that hibernacula for non-freeze tolerant treefrogs include natural tree cavities and nest boxes (Dürigen,1897, Kuhlmann and Nolte, 1986, Lachmann, 1890, Kowalewski, 1974, McComb and Noble, 1981, Snell, 1985, Johnson, 2005), burrows (Dürigen, 1897, Kuhlmann and Nolte, 1986, Lachmann, 1890, Kowalewski, 1974, Snell, 1985, Irwin et al, 1999), crevices (Dürigen,1897, Kuhlmann and Nolte, 1986, Clausnitzer, 1986, Lachmann, 1890, Kowalewski, 1974, Harris, 1975, Irwin et al, 1999), buried in mud or soil with high water content (Dürigen, 1897, Ihara, 1999), buried in dry soil (Schaub and Larsen, 1978, Reichholf, 1986), sheltered under decaying vegetation (Dürigen,1897, Kuhlmann and Nolte, 1986, Lachmann, 1890, Kowalewski, 1974, Snell, 1985, Stumpel, 1990, Iangrai, 2011), inside banana stems (Iangrai, 2011), inside man-made apparatus such as PVC tubes (Boughton et al, 2000, Zacharow et al, 2003) or finally in manmade infrastructures (Stumpel, 1990).

The Japanese Treefrog (*Dryophytes japonicus)*, synonymous with *H. stepheni* or *D. ussuriensis* in parts of its range (Dufresnes et al, 2016) and previously associated with the *Hyla* genus (Duellman et al, 2016), is widespread throughout North East Asia and is the most common amphibian in the Republic of Korea, where it mostly breeds in rice paddies (Park et al, 2013). The breeding ecology of the species is well documented (Fujioka and Lane, 1997, Hirai and Matsui, 2000, Borzée et

al, 2016a,Borzée et al, 2016b), but brumation and hibernation ecology is best described as “underground hibernation” (Sugimoto and Jiang, 2008). It is also known that the species can survive very low temperature in laboratory conditions (Berman et al, 2016). The physiological mechanisms involved in the induction of hibernating during the coldest period of the year are regulated by molecular reactions involving the cold-inducible RNA binding protein (*cirp*) (Boutilier et al, 1997), detailed by Sugimoto and Jiang (2008) for *D. japonicus*. In addition, the expression of *cirp* is induced by light signals, resulting in a higher concentration of *cirp* in the brain, eyes and ovaries in winter, for an average temperature of 8.2 ± 5.1 °C in comparison to summer average 22.4 ± 4.1 °C. Brumation is therefore mainly induced by a variation in the combination of cold stress, as demonstrated through laboratory experimentation, and secondarily by the photoperiod.

Through HDF tracking of *D. japonicus* at a brumation site, this study first focuses on: (1) does *D. japonicus* relies on shelters to maintain body temperature, and (2) does microhabitat preference change with environmental conditions during brumation in *D. japonicus*? Understanding brumation and hibernation ecology is critical from several aspects. Despite being the most abundant amphibian species in Korea, *D. japonicus* is under pressure from urbanisation and habitat encroachment throughout its range. Finally, this study is important at a much broader

scale, as even basic natural history of habitat use by brumating and hibernating anurans is largely unknown. Finally, restoration focuses on breeding habitats in amphibians, neglecting overwintering habitats. This negligence often results in the failure of restoration plans.

MATERIAL AND METHODS

Field site

The study site is a lowland forest, located in Paju, Republic of Korea (37.7512° N, 126.7272° E, roughly 7800 m^2). Male *Dryophytes* call in leks during the breeding season in rice paddies (0.25 km^2) right below the study site (Kim, 2015). The forest is typical of the secondary vegetation, composed of Chinese chestnuts (*Castanea crenata)*, Japanese red pine trees (*Pinus densiflora*) and a few Amur maple trees (*Acer palmatum)*. This type of secondary vegetation is a common replacement of the original Manchurian broad-leafed forest ecotype, composed of Korean pines (*Pinus sp.*), lindens (*Tilia sp.*), ashes (*Fraxinus sp.*), oaks (*Quercus sp.*), maples (*Acer sp.*) and elms (*Ulmus sp.*; Shannon, 1956). Korean forests were replaced by chestnut trees following large-scale habitat destruction during the Korean War (1950-53). The ecotype of this site is similar to the one used by a related species, *D. versicolor*, using cavities and tree holes in oaks as foraging sites (Mahan and Johnson, 2007).

Tracking

We caught 30 adult male *D. japonicus* in the forest at the study site on 10 September 2013: 27 on chestnut trees and three on Amur maple trees. All frogs were caught at night, aided by the presence of male advertisement calls, produced for unknown reasons. This method may have biased the individuals caught for this study, but no alternative method was available as individuals were hard to find during daytime. Treefrogs were not heard calling from any other tree species. After capture, we housed each treefrog in a separate circular PVC container 14 cm in diameter and 7 cm high until experimentation. Individuals were fed *ad libitum* with calcium and vitamin powdered crickets. Each container was set with a layer of wet but not soaked paper towels and natural oak vegetation. Individuals were set close to an open window, but not exposed to the sun, to maintain natural temperature and circadian rhythm. The day after capture, each frog was weighed (mean ± SD = 3.45 ± 3.83 g) and measured for snout-vent length (SVL; mean ± SD = 3.39 ± 0.44 cm). The procedure was repeated before release (mean ± SD = 3.83 ± 1.67 g) to assess weight change (mean ± SD = 0.38 ± 0.08 g) over 32 days in captivity. Captivity for 32 days was required for ethical reasons, it becomes unlikely to find enough individuals later in the season without destroying the habitat, and the captivity period ensured the standardisation of individuals weight gain. Twenty-eight frogs with positive weight gain

were used for the experiment, and the two individuals with no weight gain were released at the point of capture. Each frog was fitted with a schottky diode (model R2; RECCO AB; Lidingö, Sweden) coupled with an antenna tied to the frog's upper hind-legs with cotton-free gauze following the method developed by Borzée et al (2016a). The position of each individual was assessed with a Harmonic Direction Finder (HDF; RECCO AB, R2; Lidingö, Sweden).

For the tracking experiment, frogs were released maximum five at a time, until all 28 individuals were released. Release was conducted at the base of trees close to the point of capture. Individuals released at the same time were at least 15 m apart. Tracking was conducted for 72 h between 16 and 24 October 2013. Two individuals were not tracked for the full 72 h due to swollen legs (11 h 50 m and 68 h after release). Each individual location was inspected with the HDF every 129.70 ± 59.94 min (mean ± SD). At each inspection we also collected the following information: time of day, air temperature (°C), relative humidity (%; HT-350; Iondo; Seoul, Republic of Korea), luminosity (lux; Lux/Fc Light meter TM-201; Tenmars; Taipei, Taiwan), and body and substrate temperature(°C; UT-300A, Uni-Trend Technology, Dongguan China). Frogs were visually inspected every 6 h to prevent any adverse effects of the antennae on frog health (*i.e.* physical blockage).

In addition, we noted the types of microhabitat at each inspection

point. Microhabitats were divided in three categories: bark (when a frog was on a tree trunk, either on or under the bark), leafy green vegetation (when frogs were on any fully or partially leafy substrate, at any elevation except ground as it is only dead leave at that season – this included both sitting on and sitting below green leave), and ground (including dead litter). We also recorded the position of the frogs in relation to the microhabitat: above, or below the upper layer of the substrate, independently of the depth of the frog within the substrate. Splitting each category into above or below substrate resulted in individuals potentially sheltered when below bark and below ground. Finally, we recorded the tree species where the frogs were present. We also recorded the distance from the release point, which was used to determine the distance moved.

Data for eight individuals had to be discarded due to antenna bands falling off within 12 h of tracking. This problem did not occur in an earlier study (Borzée et al, 2016a), possibly due to high humidity close to rice paddies in summer, preventing the belts holding antennas from sliding off.

On-site wild frogs

Opportunistic data were collected for any on-site wild frog seen during the experiment (n = 151). While looking for the frogs tracked through HDF, we systematically checked for the presence of any frog in any wooden cavity encountered. When frogs were found, usually clustered, we collected body and substrate temperatures of a single individual for

comparison with the tracked frogs. These wild frogs were not tracked, and therefore not identified individually. Thus, there is a potential for pseudo-replication, although this is unlikely as tracked frogs typically moved considerably, and because most cavities investigated were regularly empty.

Data analysis

If a frog moved at least 5 cm from the last tracking point, then the frog was recorded as active and in displacement. This minimal distance of 5 cm is justified by the recurrent exact same position exhibited by individuals over several HDF position checks. For the 300 data points < 5 m of height, 203 individuals (67.7 %) were not found to have moved between two inspections, while 97 only (32.3 %) had moved. Besides, activities at similar "time of day" are usually repeated over days within the season and thus autocorrelated in subsequent analyses. However, very likely because the hot and cold waves during the experiment disturbed the daily pattern, this correlation was not significant in this study, and with a relatively low correlation coefficient (r = -0.17; p = 0.071). For statistical analyses the microhabitat used was nominally encoded, and the shelter and displacement variables were binary encoded. ID here refers to the identification number given to each individual. Time of day was encoded as a continuous variable. We first investigated whether particular tree species were preferentially used by *D. japonicus* through a log-linear test

with backwards elimination.

Tracking was impaired by physical restriction. That is, treefrogs above 5 m were impractical and dangerous to reach. We thus conducted a preliminary analysis to test for variations below and above 5 m. We ran a linear mixed-effects regression (LME) model with "frog location below or above 5 m" as a dependant variable. Random factors were ID, day and tree species, and covariates were time of day and air temperature (Table 1). These factors were included in the GLM, as data was not collected > 5 m for the other factors. Luminosity and humidity were excluded as they were significantly correlated with time (for all four correlation tests: $r > 0.46$; $p < 0.001$) and temperature (for all four correlation tests: $r > 0.71$; $p < 0.001$). Significant collinearity was not detected between other factors (for all factors: $0.42 < r < 0.70$; $p > 0.129$). When testing for violation of assumptions for the model, we visually verified the absence of outliers. We then determined whether the residuals were normally distributed using the Kolmogorov-Smirnov test for normality with Lilliefors Significance Correction (for all factors: $0.07 < D_{407} < 0.53$, $p > 0.05$). Finally, we determined the non-violation of assumptions through homogeneity of variances with Levene's test for homogeneity of variances for all factors (for all factors: $1.13 < F_{(1,405)} < 12.53$, $p > 0.076$). Only these factors were included for the model, as any other factor would have been missing for all data points above 5 m.

We completed tracking for 19 individuals for 72 h, which resulted in 407 data points. Of 407, there were 107 data points above 5 m. Only the points below 5 m were used for all subsequent analyses, despite the decrease in valid data points. We argue that shelters under barks are less numerous as frogs go higher and branches become narrower. Thus data above 5 m would have been important for factors such as feeding (not included in this analysis) but not for factors such as sheltering and habitat use (included in this analysis).

We investigated which abiotic variables were critical for shelter use by performing a LME model. The dependent variable was "shelter use", and fixed factors were day, and tree, while ID was set as a random variable. Covariates were temperature, humidity, luminosity, time, body temperature, and substrate temperature. Each of the factors and covariate were set as main effects in the model (Table 2). These factors were selected because the use of "shelter" was correlated with humidity (r = -0.17; p = 0.003), body temperature (r = 0.21; p < 0.001), substrate temperature (r = 0.18; p < 0.001), and also because body temperature and substrate temperature were strongly correlated (r = 0.97; p < 0.001). The correlated variables were both retained in the model, and the overall model was valid, however it is important to note that the individual coefficients are likely unreliable and may change substantially through even small changes in the value of individual data points. Significant

collinearity was not detected between other factors. Besides, no outlier was detected and the residuals were normally distributed under plot observations. Finally, variances were found to be homogeneous for all factors under a Levene's test, at the exception of the factor temperature, although maintained for downstream analyses ($F_{(1,298)} = 0.81$, $p < 0.005$).

Table A6.1. General linear model results depicting the impact of abiotic variables on vertical space use above and below 5 meters height for *Dryophytes japonicus* in Paju, Korea.

	df	χ^2	*F*	*p*	Coefficient
ID	18	2.47	34.16	< 0.001	615.03
Day	5	0.37	5.13	< 0.001	25.64
Tree	2	0.86	11.80	< 0.001	23.62
Air temperature	1	0.13	1.84	0.175	0.05
Time of day	1	0.03	0.48	0.827	1.84

We decided to ignore the heteroscedasticity as the impact on the model is generally minor. Despites this violation of assumptions in models, the least square estimates remain unbiased, and the estimates of the slope and intercept remain normally distributed as the residuals are normally distributed. The estimate for the variance of the slope may however be inaccurate, although likely insignificantly (Pinheiro and Bates, 1978, Verbeke and Molenberghs, 1997, Jacqmin-Gadda et al, 2007).

Subsequently, we answered the question of microhabitat, testing for the use of the different microhabitats in relation to biotic and abiotic variables, use through a mixed-effects regression with microhabitat as dependant categorical variable, ID, day and tree as random factors and body temperature, luminosity and height as covariates (continuous variables), with all co-factors set under main effects in the model (Table 3). Other variables, such as air temperature, substrate temperature and humidity, were not included in the model because of collinearity between variables. None of the assumptions was violated, as seen above for the same variables used for different tests. Finally, we used LSD post hoc analyses to compare variations within microhabitats. To determine factors significant for variability in body temperature during brumation, we ran an additional Generalised Linear Mixed Model using a linear link function, with body temperature as dependent variable, and microhabitat

as factor, and temperature, humidity and height as covariates.

Table A6.2. Linear mixed-effects regression model results explaining the impact of abiotic and abiotic variables on "shelter use" during brumation for Dryophytes japonicus in Korea.

	$\sum\chi^2$	*df*	χ^2	*F*	*p*	Coefficient
ID	25.59	18	1.42	9.75	< 0.001	175.47
Day	2.34	5	0.47	3.21	0.008	16.12
Tree	0.89	2	0.44	3.05	0.051	6.03
Air temperature	0.09	1	0.09	0.66	0.415	0.69
Luminosity	0.01	1	0.01	0.10	0.921	0.01
Humidity	0.08	1	0.08	0.55	0.458	0.72
Body temperature	0.53	1	0.53	3.66	0.037	3.57
Substrate temperature	0.18	1	0.18	1.24	0.266	1.23
Time	0.29	1	0.29	2.01	0.158	2.00
Error	39.05	268	0.15			

The test was ran under main effect models for each variables, and the parameters scaled under a maximum likelihood estimate. Outliers, normality of the data and homogeneity of variance has already been tested prior.

In order to characterize the relationships between vertical movement, ecological variables, and time of day, we conducted a linear mixed-effects regression with the vertical movement as the dependent variable, tree and microhabitat as random factors; and time of day, substrate temperature, luminosity, humidity and frog height as covariates. All co-factors were set as main effects in the model (Table 5). We tested for collinearity between time of day and height with a Pearson Correlation as vertical daily variation is observed in numerous species and was expected for *D. japonicus*, and all other assumptions were met, as justified above for the relevant variables. Finally, to compare the variation between body and substrate temperature between the on-site wild frogs and the one tracked, we first calculated the difference between substrate temperature and body temperature for each of the two datasets separately. We subsequently ran a Kruskal-Wallis test on the calculated values, due to the non-normal but similar variability in the distribution of the data, with on-site wild frogs or tracked individual as factor and the temperature as dependent variable. All assumptions were met for this test as the data were independent. All data was analysed with SPSS 21.0 (SPSS, Inc., Chicago,

IL, USA).

Table A6.3. Mixed-effects regression with microhabitat use as dependent variable performed to define which variables were important for the use of a specific microhabitat by *Dryophytes japonicus* in Korea.

	-2 Log likelihood	χ^2	*df*	*p*-value	Coefficient
ID	791.44	4.53	5	0.475	0.41
Day	842.02	55.11	5	< 0.001	2.59
Tree	810.12	23.21	5	< 0.001	0.96
Luminosity	828.32	41.41	5	< 0.001	0.56
Body temperature	844.07	57.17	5	< 0.001	0.82
Height	874.35	87.49	5	< 0.001	0.06

RESULTS

Tree use

Out of 407 position points, there were 271 records of *D. japonicus* in chestnut trees, 26 records in maple trees and three records in pine trees. Thus, tree use was significantly different among tree species. The log linear test was a perfect fit to the data as it reached saturation, with K-way and higher order effect reaching K = 2 for backward elimination before losing significance (Likelihood Ratio; $\chi^2 = 234.36$; $df = 4$; $p < 0.001$). The backward elimination model with the higher effect, although negative, was chestnut*maple*pine ($Z = -3.95$; $p < 0.001$), while the second best one was chestnut*pine, although not reaching significance ($Z = 1.69$; $p = 0.092$). This indicates that for the three tree categories, *D. japonicus* was significantly more often found on one tree species than the others.

Habitat use and heights

Out of 19 individuals, eight did not go above 5 m during tracking. Furthermore, no frog went above 5 m on the first day of tracking, and no individual was observed in pine trees above 5 m. A total of 90.3 % of observations were in chestnut trees, which were also predominantly used, in 95.3 % of observations, above 5 m. Maple trees, were used in 8.7 % of observations above 5 m but in 4.7 % of observations below 5 m.

Table A6.4. Descriptive statistics for microhabitat use and the relation with tree species and total use. *Dryophytes japonicus* had a clear preference for chestnut trees and was found in majority on leafy vegetation.

Microhabitat	Chestnut	Maple	Pine	Total	Frequency
On tree bark	28	1	0	29	9.70
On leafy vegetation	83	0	2	85	28.43
On ground	46	2	1	49	16.39
Below tree bark	54	6	0	60	20.07
Below leafy vegetation	52	16	0	68	22.74
Below ground	8	0	0	8	2.68
Sub-total	271	25	3	299	
Frequency	90.64	8.36	1.00		
Air temperature (°C)	9.87	10.33	11.17		
SD	4.89	3.97	3.23		

The median value of luminosity was 46.5 ± 9436 lux (min = 1, max = 56000) for observations below 5 m, while it was 240 ± 8757 lux (min = 1, max = 46600) for observations above 5 m. This shows that treefrogs were > 5m at times of day with greater luminosities, suggesting basking behaviour at higher heights. The results of the LME model for above 5 m showed that ID, day, tree, and luminosity were significant (Table A6.1).

Shelter use

Body temperature was the only significant fixed effect in the shelter-use LME model. Mean body temperature for tracked frogs was 10.88 ± 4.25 °C (mean ± SD) when sheltered (n = 154), versus 8.90 ± 5.13 °C when not sheltered (n = 146; Fig. A6.1; LME model; $\chi^2 = 0.63$; $df = 1$; $p = 0.039$). The minimum body temperature was 2.10 °C when sheltered and 1.10 °C when not sheltered. The maximum body temperature was 24.50 °C when not sheltered, but it was 20.90 °C when sheltered. This shows that *D. japonicus* were able to use shelters to regulate body temperature. Interestingly, time of day was not significant for shelter use, likely due to a cold wave on the third day of the experiment: Pearson correlation coefficient between day and time of day: $r = -0.22$, $n = 407$, $p < 0.001$; and significant different temperature between days: one-way ANOVA; $F = 10.86$, $df = 6$, $p < 0.001$; with mean temperature (± SD) day 1 = 8.77 ± 0.30, day 2 = 11.45 ± 4.06, day 3 = 6.98 ± 3.09, day 4 = 13.20

± 6.04, day 5 = 12.59 ± 5.27; day 6 = 12.33 ± 4.12.

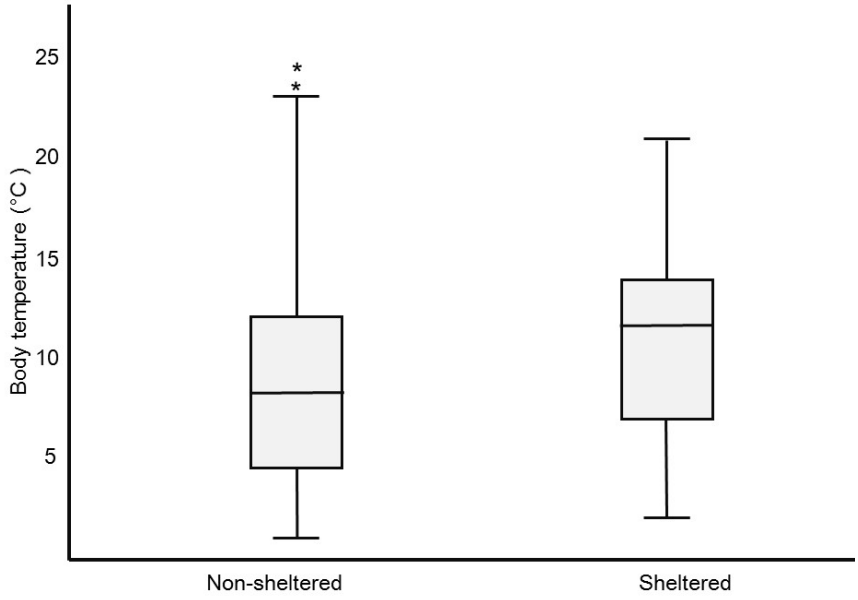


Figure A6.1. Variation in body temperature between sheltered (n = 154) and non-sheltered individual (n = 146) *Dryophytes japonicus* during brumation in Korea. The line within the box represents the mean; the top and bottom lines represent 75 and 25 percentiles of the data, respectively; top and bottom whiskers represent 95 and 5 percentiles, respectively; asterisks represent outliers. The body temperature was significantly different between the two groups.

The cold temperatures may have forced tracked frogs to rely on shelters more often and for longer than at other times. The other significant factors of the LME model were random effects: ID and day (Table A6.2).

Microhabitat use

The microhabitat use-GLM indicated a predominant use of leafy vegetation in chestnut trees as microhabitat. Treefrog use of individual types of microhabitats ranged from 9.7 to 28.3% of total (*i.e.*, 100%) of observations, with the exception of "buried under tree bark", which accounted for 2.7 % of observations. However, the distribution for microhabitat use in relation to plant species was skewed towards leafy vegetation and chestnut trees (Fig. A6.2). The mixed-effects regression performed to ascertain microhabitat use in relation to environmental factors were statistically significant for luminosity, body temperature, height, day and tree species (Table A6.3). The model was significant ($\chi^2_{(30)}$ = 288.15, $p < 0.001$ and explained 63.5 % (Nagelkerke R^2) of the variance. Tracked treefrogs were most frequently found on chestnut trees (90.64 %), followed by maples trees (8.36 %) and pine trees (1.00 %; Table A6.4). Although the ranges were widely overlapping, body temperature was significantly different among microhabitats (Fig. A6.3). When below leafy vegetation, body temperature was highest (11.14 °C ± 4.38), while being on top of leafy vegetation was associated with the

lowest temperature (8.15 °C ± 5.48; Table A6.4).

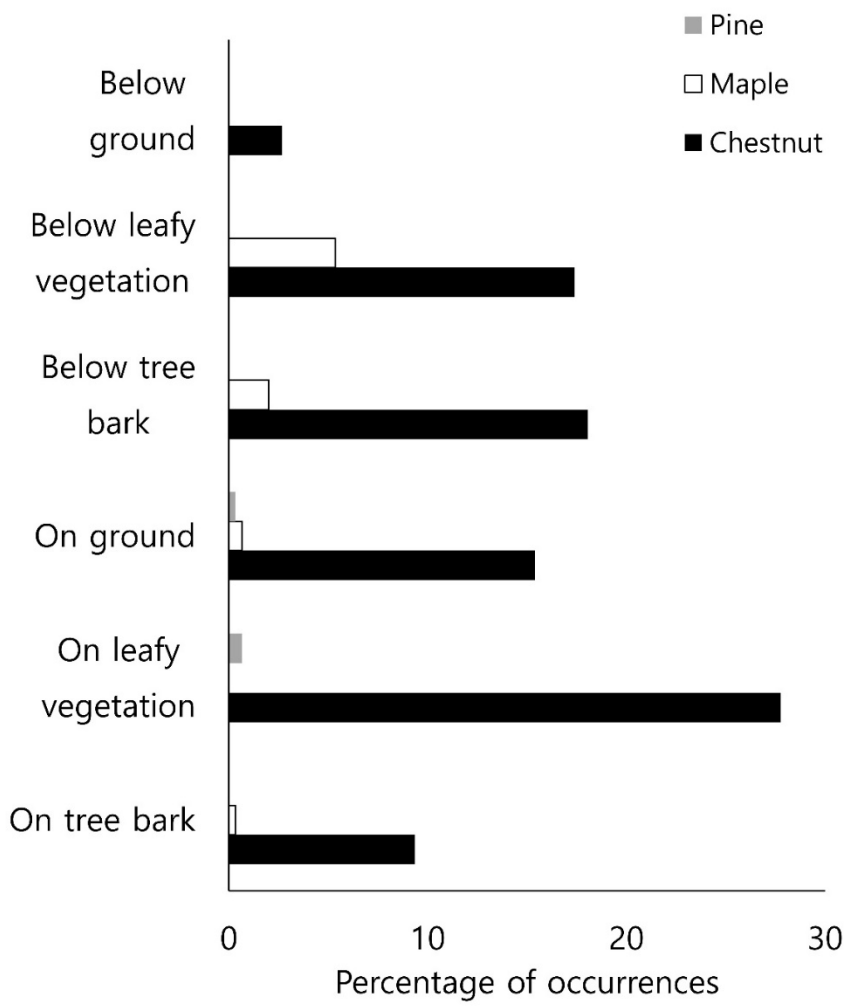


Figure A6.2. Tree species and microhabitat use by *Dryophytes japonicus* in Korea. 100 % of occurrences matches corresponds to the sum of all values for each of the three tree species and the six microhabitats.

Besides, LSD post hoc analyses showed that "on leafy vegetation" was significantly different from "on ground ($p = 0.008$), "under tree bark" ($p < 0.001$), and "under leafy vegetation" ($p < 0.001$).

The GLMM testing for the factors explaining variability in body temperature during brumation was significant (Omnibus test; Likelihood ratio $\chi^2_{(8)} = 392.37$, $n = 300$, $p < 0.001$). Microhabitat ($\chi^2_{(5)} = 16.57$, $p = 0.005$), temperature ($\chi^2_{(1)} = 119.17$, $p < 0.001$) and humidity ($\chi^2_{(1)} = 69.45$, $p < 0.001$) were significant, while height was not ($\chi^2_{(1)} = 0.88$; $p = 0.346$).

Movement

During tracking, frogs were found moving in average 9.23 (± 10.55) m away from the release point. The individual moving the most moved 39.80 m in 72 h. Individuals were mostly found staying in the vicinity of the same tree, or bush, but some individuals were found changing trees, mostly by climbing down, moving over the leaf litter, and climbing up another tree.

The results of the linear mixed-effects regression for movement showed that microhabitat and time of day were significant. Treefrogs were most active when on leafy plants, and least active when underground (Fig. A6.4). Frogs were generally active in the late morning (mean = 11:22; 95 % confidence interval: 10:31 to 12:13), while on average, more active in the early afternoon (mean = 12:38; 95 % confidence interval: 11:08 to 14:08).

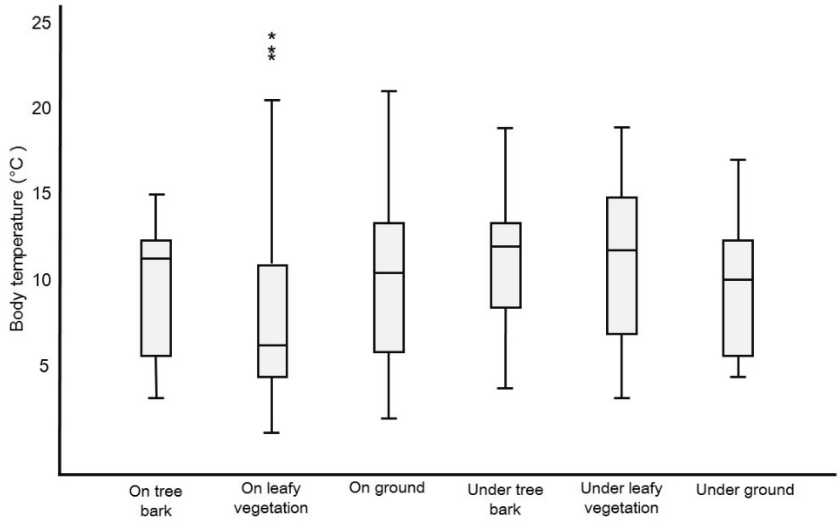


Figure A6.3. Microhabitat use and body temperature during brumation for *Dryophytes japonicus* in Korea. The minimum and maximum temperatures were recorded when the individuals were on leafy vegetation and on the ground, respectively (below ground: $n = 8$, below leafy vegetation $n = 69$, below tree bark $n = 60$, on ground $n = 49$, on leafy vegetation $n = 85$, on tree bark n = 29). The line within the box represents the mean; the top and bottom lines represent 75 and 25 percentiles of the data, respectively; top and bottom whiskers represent 95 and 5 percentiles, respectively; asterisks represent outliers. LSD post hoc analyses showed that "on leafy vegetation" was significantly different from "on ground ($p = 0.008$), "under tree bark" ($p < 0.001$), and "under leafy vegetation" ($p < 0.001$).

Notably, frog height was not a significant predictor of frog movement (20.59 ± 58.20; 19.56 ± 55.90; range = 0 to 420 cm, for active and inactive, respectively). The relationship between height and time of day was not significant (r = -0.06, n = 300, P = 0.310).

Wild frogs

The difference between substrate and body temperatures for wild frogs was 0.12 °C (± 0.90), and for tracked frogs was 0.06 °C (± 1.2). This difference was not significant (Kruskal-Wallis test; χ^2 = 13.21; df = 16; p = 0.657), meaning the thermoregulation of the tracked treefrogs was similar to that of the wild ones. It is however interesting to see that the ranges of substrate temperature (1.10 to 24.50 °C) and body temperature (1.50 to 28.30 °C) of the tracked treefrogs seemed to be wider than the substrate temperature (2.50 to 18.70 °C) and body temperature (2.90 to 18.10 °C) of the wild frogs.

DISCUSSION

Our tracking study demonstrates that *Dryophytes japonicus* moved between microhabitat following environmental variations, and especially based on temperature. This selected exploitation of the habitat is expected to allow frogs maintaining their temperature during the brumation period.

Table A6.5. Linear Mixed-effects Regression Model with movement as dependent variable. *Dryophytes japonicus* was in movement at specific microhabitats and height only.

	Sum of Squares	*df*	χ^2	*F*	*p*-value
Tree species	0.70	2	0.05	0.29	0.151
Microhabitat	7.14	5	1.42	8.18	< 0.001
Time of day	1.28	1	1.28	3.93	0.009
Body temp.	0.05	1	0.05	0.26	0.610
Luminosity	0.01	1	0.01	0.03	0.886
Humidity	0.67	1	0.67	3.65	0.057
Height	0.14	1	0.14	0.75	0.388

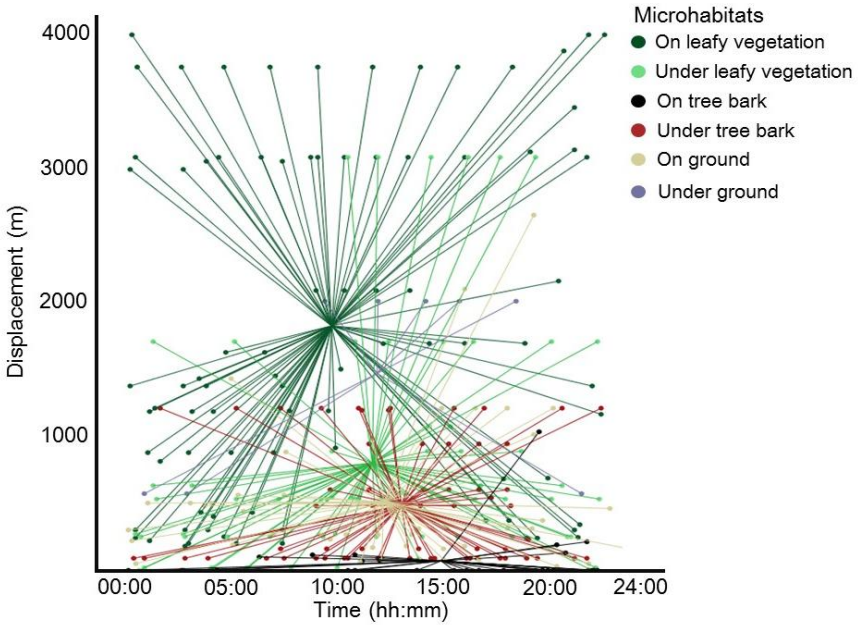


Figure A6.4 Displacement in relation with time of day for each microhabitat used by *Dryophytes japonicus* during brumation in Korea. All points from the same microhabitats share a centroid (rounds markers). The location of centroids represent the average time of day at which activity occur for a given microhabitat, and indicates an increase in activity in the early afternoon (below ground: n = 8; below leafy vegetation: n = 69; below tree bark: n = 60; on ground: n = 49: on leafy vegetation: n = 85; on tree bark: n = 29). Some aspects of the graphics might only be fully comprehensible in the PDF version where they are reproduced in colour.

Like other ectotherms, *D. japonicus* relied on behavioural thermoregulation by means of shelter use (Lambrinos and Kleier, 2003).

The analysis of microhabitat preference for brumation for *D. japonicus* lead to a clear distinctions in microhabitat in relation with temperature and movement. *Dryophytes japonicus* visited shelters when body temperature dipped below an average of 10.2 °C. The treefrogs were most active when on the "leafy vegetation" microhabitat", and in majority *circa* 10 am in the morning. Displacement distances decreased in the early afternoon for the other microhabitats "under leafy vegetation", "under bark" and "on the ground". Around 3 pm, the microhabitat mostly used was "on bark", with displacements almost totally halted. This behavioural thermoregulation is likely to delay the beginning of hibernation and save the body fat reserve acquired after the breeding season (Mayhew, 1968). Besides, the correlation between shelter use and body temperature suggests that treefrogs are intermittently active, and therefore in a state of brumation, which makes the onset of hibernating a gradual process, hypothetically during the build-up of the RNA binding protein *cirp* molecules (Sugimoto and Jiang, 2008).

The behaviour of the tracked frogs did not seem to differfrom that of wild frogs. The two groups had similar thermoregulation outcomes, suggesting that the tag used for tracking did not affect their ability to thermoregulate during the course of the study. Activity at higher

temperatures, *i.e.* afternoon, may relate to feeding as enzymatic activity required for digestion is correlated with temperature (Harlow et al, 1976), and sun-bathing provides the required heat (Schneider, 1977, Rodríguez-Prieto and Fernández-Juricic, 2005). However, the wider temperature range covered by tracked individuals may pertain to tracking efficiency. When cold, wild frogs will be deep in their hibernaculum and may not be seen, while when relatively warm, they may go up the trees for foraging and would therefore not be recorded either. However, the study was conducted too early in the season to see a threshold temperature, defined here as the point at which the temperature induce immobility in to the species. This threshold temperature is difficult to predict as reportedly widely different between species, such as illustrated by *Rhacophorus arboreus* being active from about 7 °C (Ito and Fukuda, 2007) or *Polypedates leucomystax* and *R. bipunctatus*, hibernating between 13.5 °C and 20.5 °C (Iangrai, 2011).

During the tracking period, the treefrogs were most likely found in chestnut trees, although pine and maple trees were available in the study site. This association with chestnut trees could be related to the size of the trees. Chestnut trees made up most of the upper canopy of the forest and therefore boast wider trunks and branches, where tree holes are more likely to occur. Treefrogs typically use tree holes as shelters or hunting grounds (Mahan and Johnson, 2007, Johnson et al, 2008). Additionally,

chestnuts at the site had loose bark on their trunks, which individuals could shelter beneath, whereas the pine and maple trees did not have loose bark. Alternatively, the lower acidity associated with pine trees might deter treefrogs from their use (Gosner and Black, 1957, Fairman et al, 2013).

Our study was not able to discriminate *D. japonicus* as a freeze tolerant species, but the species follows the pattern described by freeze intolerant species, and appears to use underground burrows to achieve thermal insulation during freezing periods (reviewed by Irwin et al, 1999). However, we want to acknowledge that despite location points being independent in this study, the results might be improved by modeling for autocorrelation. Another possibility would be supercooling, such as the cricket frogs (*Acris crepitans*) that can supercool when dry and survive negative temperatures without freeze resistance mechanisms, *i.e.* mean supercooling point at –5.5 °C; ranging from –4.3 to –6.8 °C (Irwin et al, 1999). The least frequently used microhabitat for shelter was underground, and no frog was found sheltering at the bottom of any water body. Thus, it is impossible to reject hyper cooling by *D. japonicus*, although unlikely, and the species is also unlikely to hibernate underwater at our study site.

The association between body temperature and microhabitat use was also related to the activities of individual frog. Higher temperatures

allow treefrogs to feed. Thus we predict that individuals with higher body temperatures will be seen in microhabitats rich in preys. Similarly, treefrog movements were related to microhabitats, time of day and height, representative of the different requirements for hunting and sheltering. Consequently, we predict that leafy plants, where most daytime frog activity occurred, will be rich in preys. Frogs were least active when underground, suggesting sheltering. Based on these results and predictions, we suggest that *D. japonicus* was actively foraging and/or sunbathing in the early afternoon, the warmest time of the day, and carrying out other behaviours at other times of the day.

During tracking, we observed wild treefrogs in clusters of two to seven individuals, and one of the frogs tracked was found within one of those clusters twice. However, no social behaviour apart from mating calls are known for Palearctic treefrogs (Wells, 1977, Wells and Schwartz, 2007, Wells, 2010). A simple explanation to this observation could be the absence of adequate hibernating hides, while the density of treefrogs was relatively high at our study site.

Finally, and in relation with the possible over-crowding of hibernaculum, two individuals were recorded entering the insulation cavities in the bricks of a barn on the edge of the research site. These observations imply that *D. japonicus* can use human infrastructures as shelters, which in turn could explain the abundance of the species, in

comparison to the endangered *D. suweonensis*. Furthermore, conservation of amphibians is most often related to their breeding site (Lanoo, 2006), and habitat modification and lack of over-wintering sites could have negative impacts as serious as the filling of breeding wetland for the species.

PART III: PHYLOGENETICS

This part contains all aspects of phylogenetics in relation with the endangered status of amphibians. This includes hybridisation and species reversal (chapter 9), the determination of the species status (chapter 10) and the description of mitogenome for a species (appendix 7).

Chapter nine

LARGE-SCALE HYBRIDISATION AS AN EXTINCTION THREAT TO THE SUWEON TREEFROG (*DRYOPHYTES SUWEONENSIS*).

ABSTRACT

Amphibians are in the midst of a sixth mass extinction, and human activities play a major role. Reasons are multiples and intertwined, including habitat loss, diseases, and hybridisation. Hybridisation and introgression of previously isolated species potentially leads to speciation reversal, through which the genepool of two species are merged, or the genepool of a species is incorporated into another one. This research aims at demonstrating that the large scale hybridisation of the endangered *Dryophytes suweonensis* with the numerous *D. japonicus* may lead to the extinction of the species with the smallest population size. Based on the analysis of COI mtDNA sequences for 404 individuals from 35 populations and six polymorphic microsatellites for 381 individuals from 34 populations, we demonstrate a generalised, bi-directional and geographically widespread hybridisation of the two species. Evidence of fertile back-crosses is provided by relatively high numbers of individuals in cyto-nuclear disequilibrium, and the presence of hybrid individuals twice the distance of species' distribution-width away from the distribution limit. We recommend the preservation of pure *D. suweonensis*

populations or ex-situ conservation for the survival of the species.

INTRODUCTION

Threats to species are numerous, with most linked to human activities (Forester and Machlist 1996; Liu et al. 1999; Pereira et al. 2010). For indirect threats such as hybridisation, as species are commonly brought into contact because of anthropisation of the environment (Rhymer and Simberloff 1996; Allendorf et al. 2001; Olden et al. 2005; Laikre et al. 2010; Casas et al. 2012). When two recently diverged species are brought back in contact, the phenomenon is referred to as reverse speciation (*sensu* Seehausen 2006), albeit not a "reversal" *per say*. One example of reverse speciation due to human modification of the environment was found in whitefish in Switzerland, where eutrophication due to human activities lead to hybridisation and speciation reversal (Vonlanthen et al. 2012).

Widespread hybridisation negatively impacts species identity as introgression erodes the genetic difference between two species, until the two species collapse into a hybrid swarm where parents and hybrids can interbreed (Vonlanthen et al. 2012). Such is the case between the mallard duck (*Anas platyrhynchos*) and New Zealand grey duck (*A. superciliosa superciliosa*), where the latter species is under threat of extinction because

of introgressive hybridisation (Wiegand 1935; Rhymer et al. 1994). Another example involving amphibians is within the hybridogenetic *Pelophylax* complex in Europe, where introduced species (*P. ridibundus* in Northern Europe and *P. esculenta* in Spain) hybridise with local species. Such hybridisation pushes the local species towards extinction - *P. esculentus* and *P. lessonae* (Holsbeek and Jooris 2010), and *P. perezi* (Arano et al. 1995), respectively. The negative effects of hybridisation are diverse, with extreme cases leading toward the loss of a species and a reduction of biodiversity. Less extreme, but also having negative impacts on species are outbreeding depression and decreased in fitness.

The Korean Peninsula is populated by two species of treefrogs: *Dryophytes suweonensis*, originally described as *Dryophytes suweonensis* (Kuramoto 1980; Duellman et al. 2016) and *D. japonicus* (Yang and Park 1988b; Yu and Lee 1989; Lee and Park 1992; Riehl et al. 1995; Yang et al. 1997; Lee et al. 1999; Chun et al. 2012; Hua et al. 2012). The two species differ in call (Kuramoto 1980, Jang et al. 2011, Park et al. 2013), morphometrics (Kuramoto 1980; Borzée et al. 2013), and behaviour (Borzée et al. 2016a; Borzée et al. 2016b). The divergence between clades of the "*D. japonicus* group", including these two *Dryophytes* species and *D. immaculatus*, led to the split between *D. japonicus* and *D. suweonensis* between 6.4 and 5.1 mya (Li et al. 2015; Dufresnes et al. 2016).

The two species are sympatric between the Imjin (IUCN 2017a)

and Mankyeong Rivers (Borzée et al. 2016c; Borzée et al. 2017b), with some potential populations in the Democratic People's Republic of Korea (Chun et al. 2012). *Dryophytes japonicus* is distributed throughout most of Northeast Asia and a species of least concern (IUCN 2017a), while *D. suweonensis* is restricted to the Korean Peninsula and not known to currently occur in allopatry (Fig. 9.1; Roh et al. 2014; Borzée et al. 2017b) and is listed as endangered (Ministry of Environment 2012; IUCN 2017a). The two species are able to hybridise in laboratory conditions (Kuramoto 1984), but hybrids have so far not been documented in the wild.

The probability of hybridisation increases with three types of anthropisation of the habitat (Rhymer and Simberloff 1996). (1) Local habitat modification, referred to as "hybridisation of the habitat" (Anderson 1948; Whitmore 1983.), as exemplified for Hylids (Lamb and Avise 1986; Schlefer et al. 1986), (2) regional habitat changes allowing geographic expansion of a clade within the range of the other, and (3) the creation of corridors leading to the continual movement of a clade within the breeding habitat of the other. All three types of modification are common within the range of *D. suweonensis*: (1) the centre of the range of *D. suweonensis* is slowly modified due to urbanisation (Borzée et al. 2015a), (2) agricultural development led *D. japonicus* further west than the clade was hypothetically originally distributed (Borzée et al. 2017b) and (3) agricultural development of all valleys resulted in optimally

connected environment for dispersion.

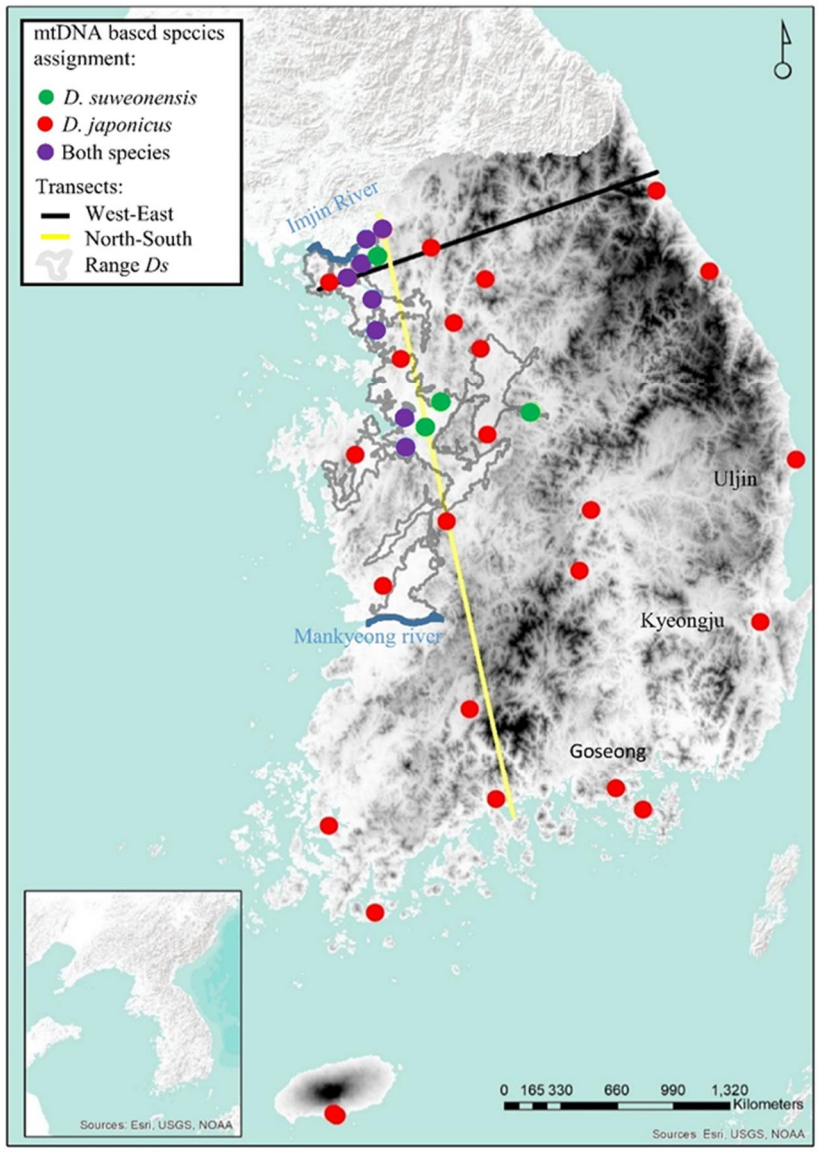


Figure **9.1.** Sampling localities for all *Dryophytes* individuals. Despite having sampled *Dryophytes suweonensis* only at some sites, later independent aural surveys indicated the presence of *D. japonicus* as well. The two transects for subsequent analyses are added to the map. Service Layer Credits: Sources: Esri, USGS, and GeoServicesMap Esri Korea.

Map generated in ArcMap 10.5 (Esri, Redlands, USA).

In this study we uncover a threat to the species integrity and survival of *D. suweonensis* due to widespread introgression with *D. japonicus*, as a result of anthropisation of the environment. Namely, the modification of wetlands and valleys into rice paddies over the last 7000 years (Fuller et al. 2007; Fuller et al. 2008) has led to colonisation new environments by *D. japonicus*, resulting in widespread bi-directional introgression between the two species.

MATERIAL AND METHODS

Field work

When this project started in 2013, very little was known about the range and ecology of the species (Kim et al. 2012a; Kim et al. 2012b). Sampling was conducted under blind conditions, aiming at the few known populations (Roh et al. 2014). Field work was conducted following a rough grid with 20 km between each sampled population over the range of *D. suweonensis,* such as estimated in 2013. Once out of the estimated range of *D. suweonensis*, sampling sites were selected every 60 km to allow for a proper representation of the potential introgression patterns within the range of *D. japonicus*. Sampling in 2013 included 404 individual from 35 sites (Fig. 9.1). Sampling sites were a minimum of 12

km apart, ensuring independence of samples as hylids disperse two to four km per year (Smith and Green 2005; Angelone and Holderegger 2009). Samples were collected under the authorisation of the Ministry of Environment (number 2013-16). Genetic material was acquired through buccal swabs, frozen within 24 h at -20˚C (Broquet et al. 2007).

Molecular work

DNA was extracted with the Enzynomics Genomic DNA Extraction Kit (Daejon, Republic of Korea) following the recommendation of the manufacturer (Genomics Extraction Kit protocol V2013-1). All DNA samples were adjusted to 40 ng/µL before PCR reactions. PCR targeting a 579 bp of mtDNA Cytochrome c oxidase subunit I (COI) were performed on a PTC-100, BIO-RAD thermocycler (California; USA) following the protocol of Jang et al. (2011) using primers LepF1 (5'-ACC AAT CAT AAA GAT ATT GGT-3') and LepR1 (5'-CCT CTG GGT GTC CGA AAA ATC A-3'). Samples were sent to Macrogen Inc. (Seoul, Republic of Korea) for direct sequencing with both forwards and reverse primers on an ABI PRISM 3100 automatic sequencer (Applied Biosystem Inc., USA).

A total of nine microsatellite markers were tested (Colgan et al. 1999; Bossuyt and Milinkovitch 2000; Bonacum et al. 2001; Biju and Bossuyt 2003; Crawford 2003; Chiari et al. 2004; Hoegg et al. 2004; Faivovich et al. 2005; Wiens et al. 2005). Six of these polymorphic

markers were successful in cross-amplifying for the two hylid species (Annex 9.1). As this project aims at determining the impact of hybridisation on the survival of *D. suweonensis*, and not to conduct a phylogenetic analysis, we considered the markers selected here to be adequate to accurately depict the genetic pattern studied. This is supported by researches reporting that as little as five microsatellites markers can be representative of the genetic structure of a population (Evanno et al. 2005; Wright et al. 2014).

PCR reactions for all microsatellites were set with the same volumes of reagent but different annealing temperatures (Annex 9.2). We used the Takara PCR Buffer (initial concentration 10X; containing 2 mM $MgCl_2$), dNTPs (10mM), labelled primers (HEX or FAM; at 10µM) and the Takara Taq polymerase (concentrated at five units/µL). Amplifications were carried out on an PTC-100, BIO-RAD (California; USA) thermocycler programmed at 94°C for 1 min, followed by 39 cycles at 94°C for 30 sec, with the specific annealing temperature for 1 min (Annex 9.2), 72°C for 1 min, and then a terminal elongation at 72°C for 5 min. PCR products were examined on a standard agarose gel at 1.5 % before being sent for genotyping and allele scoring to Macrogen (Seoul, Republic of Korea). In case of inconsistences, allele scoring was verified using the microsatellites plug-in (Biomatters Ltd) in Geneious (v9.04, Biomatters Limited, Auckland, New Zealand).

mtDNA analyses

We used DnaSP (Librado and Rozas 2009) to compute haplotype diversity, Fu's F and Tajima's D to test if the sequences selected were under selection or at mutation-drift equilibrium (Tajima 1989; Fu 1997). We then created a haplotype network in TCS with a fixed connection limit at 500 steps (estimated) and all other parameters set as defaults (Clement et al. 2000).

The phylogenetic relationship between species was inferred through the construction of phylogenetic trees using three different methods. For all trees, we used *Hyla chinensis* (GenBank accession number AY458593) as outgroup. This choice was made as the species is an outgroup, while *Hyla* is the closest related genus to *Dryophytes* (Duellman et al. 2016).

First, we used Neighbour-Joining (NJ) to build a tree with the Geneious tree builder plug-in (Biomatters Ltd), applying Jukes-Cantor genetic distance model. We choose the bootstrapping option with a 100,000 replicates, to create a consensus tree with support at 50% threshold. Second, we generated two Maximum Likelihood (ML) phylogenetic tree. This duplicate analysis was conducted to assess the robustness of the tree topology. Besides, the two analyses use different hill-climbing search methods potentially resulting in differences in tree scoring, and variation in results (Sundberg et al. 2008). The first ML tree

was built using the GTR model through the PhyML plug-in for Geneious (Lefort and Biomatters Ltd; Guindon and Gascuel 2003b; Guindon et al. 2010). We chose an estimated gamma distribution parameter and used the combined subtree pruning and regrafting (SPR), plus nearest neighbour interchange (NNI) options for tree improvement. All other parameters were set as default, with optimisation for topology, branch length and substitution rate. Bootstrap values were based on a 100,000 resampled dataset (Guindon and Gascuel, 2003). Last, we generated a RAxML tree (Geneious plugin; RAxML 7.2.8) with a GTR-GAMMA nucleotide model with 100,000 bootstrap replicates, and all other variables set to default (Stamatakis 2014).

Microsatellites analysis

We tested for the occurrence of null alleles or allelic dropout through the use of Microchecker v. 2.2.3 (Van Oosterhout et al. 2006), using Bonferroni correction. We assessed the deviation from Hardy Weinberg equilibrium with GENEPOP v 4.2 (Rousset 2008) through a U-test for each locus. We then calculated F-statistics based on the six loci through FSTAT v.2.9.3.2 (Goudet 1995), with default parameters for all but two: we chose the rarefaction option to standardise the average estimated number of alleles per locus and thus remove the bias for populations with small sample sizes (Leberg 2002), and the Bonferroni corrections to address the problem of multiple comparisons.

We then used descriptive F-statistics to obtain the range of alleles sizes, the number of alleles, the allelic richness and Nei's estimation of observed and expected heterozygosity per locus (Nei 1987). Due to the possible hybridization, the dataset was divided in three sub-sets based on mtDNA: populations with *D. suweonensis* haplotypes only, *D. japonicus* haplotypes only, and populations with both haplotypes. Here haplotype is used to define the groups as there was no overlap in haplotype between the two clades (Fig. 9.2). This *ad-hoc* analysis was conducted through tests based on 10,000 randomizations using F_{IS} statistics for Hardy Weinberg equilibrium (HWE) within-samples. SPSS v.21 (SPSS, Inc., Chicago, IL, USA) was used to statistically assess the variation between species.

For each locality with at least six individuals, we tested for a recent bottlenecks with a one-tailed Wilcoxon tests and Bottleneck v1.2.02 (Cornuet and Luikart 1996; Broquet et al. 2010). The parameters were set to the Two-Phase Model (TPM) with 95% single-step mutations and 5% multiple-step mutations, ran for 1000 iterations.

Then, to evaluate the genetic structure of populations through the allelic content of haplotypes and frequencies, we performed Analyses of Molecular Variance (AMOVAs) among populations and individuals in IMa2 with 1000 permutations (Hey 2010b; Hey 2010a). These analyses

were executed for populations with a minimum of five individuals as a low population size induces a bias in the analysis.

Patterns of genetic structure and the extent of hybridization between these two species were examined through the Bayesian clustering algorithm implemented in STRUCTURE v. 2.3.4 (Pritchard et al. 2000; Pritchard et al. 2007).

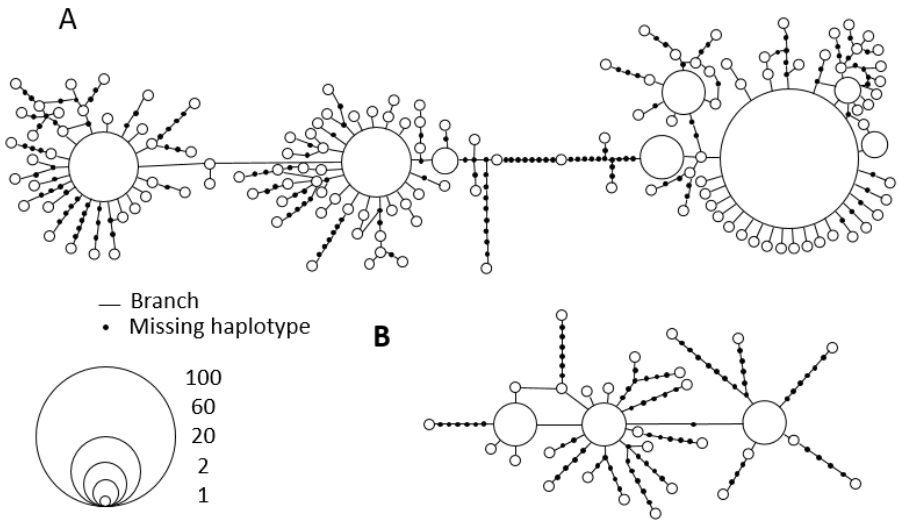


Figure 9.2. Haplotype network for (A) *Dryophytes japonicus* (*n* = 317) and (B) *D. suweonensis* (*n* = 87). The figure was drawn in TCS with a fixed connection limit at 500 steps (estimated) and all other parameters set as defaults. The star networks indicate pronounced population expansion (both species) while the missing haplotypes are indicators of local extinctions (*Dryophytes suweonensis*).

All analyses used data from the six microsatellites loci and parameters were set to the default values provided by the program (Pritchard et al. 2007). For each analysis we tested a range of clusters ("K") from one to the number of sampling sites plus three to detect the "K" best fitting our data. The best fitting "K" was detected using the posterior probabilities ("LnP(D)") for "K" obtained in the analyses and inferred from the method developed by Evanno et al. (2005), where ΔK is a summary statistic based on the rate of change of the likelihood distribution between successive values of K. The run with the highest log likelihood indicated the inferred number of populations within each species. We ran STRUCTURE ten times for each K with a burn-in of 10'000 steps followed by 500,000 MCMC steps (Evanno et al. 2005), and averaged results for the ten runs for each K..

Once K was defined, the populations included in the analysis were reordered geographically from north to south, and west to east over the estimated contact zone. We selected a North-South transect along the low plains of Western Korea as potentially matching the ecological requirements of the species (Roh et al. 2014; Borzée et al. 2017b), and West-East crossing the Baekdudaegan Mountain range, as *D. suweonensis* is not found above 100 m of altitude and presence of hybrids at higher altitude would have conservation benefits. We also ran separate STRUCTURE analyses on "pure" mtDNA *D. suweonensis* and "pure" *D.*

japonicus populations in order to test for cryptic intraspecific population structure and determine relationship between populations of the same clade.

Identification of potential hybrids

Introgression occurs when the genetic information of a clade is integrated into the genome of another following hybridisation and subsequent backcrossing (Bryson Jr et al. 2014), therefore resulting in tree incongruences when species are sympatric (Joly et al. 2009). First, hybrid individuals were identified through their assignment to more than a clade through STRUCTURE (90% threshold). Any individual with less than 90% assignment probability to a clade was considered a hybrid. We then assessed the presence of hybrids through cytonuclear disequilibrium, *i.e.* inconsistency between mtDNA and nuclear assignment. An individual was considered a hybrid if assigned to one clade by STRUCTURE but displaying the mtDNA from the other clade (Kartavtsev 2013). Cytonuclear disequilibrium was also analysed for directionality of hybridisation.

RESULTS

mtDNA: estimation of genetic variables

Sequences were recovered for all 404 individuals. Forward and reverse reads were assembled for each individual, checked by eye, and

then aligned using the MUSCLE alignment plug-in in Geneious, with a maximum of ten iterations. The sequences were first analysed through DnaSP (Librado and Rozas 2009), and 564 bp were used for analysis, among which there were 199 polymorphic sites. The average number of pairwise nucleotide differences (k) was 26.31 and the nucleotide diversity (Pi) 0.046. The total number of haplotype (h) was 168, with a haplotype diversity (Hd) of 0.94 (variance < 0.001; Table 9.1). The analysis for the two species separately was based on 317 sequences for *D. japonicus* and 87 sequences for *D. suweonensis* (Fig. 9.1). Further analysis for *Dryophytes japonicus* resulted in Tajima's D = -2.38 ($p < 0.01$) and Fu's F = -253.80, while Tajima's D = -2.42 ($p < 0.001$) and Fu's F = -29.35 for *D. suweonensis*. The negative and statistically significant D for *D. suweonensis* indicated an excess of rare mutations, potentially related to a population bottleneck, while not significant for *D. japonicus*. The negative Fu's F for both species indicated an excess number of alleles and therefore population expansion for both species, although *circa* ten times higher for *D. japonicus*. The networks between the two species were not linked, and each of the two species displayed a clear structure (Fig. 9.2).

Haplotypes for 317 individuals were within the network for *D. japonicus*. There were 36 haplotypes between the two extremities of the network, including 24 missing haplotype. The network was divided in three star-shaped sub-network, with 18 of the missing haplotypes between

two of the three sub-networks (Fig. 9.2A).

Table 9.1. F-statistics analyses conducted locus and averaged for all six loci. *P*-values are based on T-tests for difference between *Dryophytes japonicus* (*Dj*) and *D. suweonensis* (*Ds*).

	All populations	*Dj* populations	*Ds* populations	*p*-value
Gene diversity (He)	0.280	0.246	0.290	0.746
number of alleles (Ae)	2.564	2.120	2.167	0.928
Allelic Richness (AR)	1.267	1.231	1.281	0.700
Inbreeding (F_{IS})	0.576	0.620	0.369	0.251

The network for *D. suweonensis* included 87 individuals and was similar to *D. japonicus* in the way that there were two star-shaped sub-networks, although these were principally hollow, *i.e.* 15 missing haplotype between the two most distance haplotypes, 20 single mutations away (Fig. 9.2B). The start-shaped subnetworks were relatively smaller for *D. suweonensis* than *D. japonicus*, reflecting less emphasized characteristic signature of population expansion, while the hollow in the network is characteristic of species with repeated local extinction.

mtDNA phylogenetic analyses

The three phylogenetic trees were congruent and all three provided supported bootstrap values for three main divisions (Hillis and Bull 1993), with *D. suweonensis* and *D. japonicus* being reciprocally monophyletic (Fig. 9.3). Sub-structuring was detected by the PhyML (Fig. 9.3C), bisecting the *D. japonicus* clade. Interestingly, this division of the *D. japonicus* clade was not detected by the NJ tree (Fig. 9.3A) and RaxML trees (Fig. 9.3B). The haplotype networks and phylogenetic trees agreed on the assignment of individuals to specific clades, with *D. suweonensis* detected at twelve out of 35 sampling localities (Fig. 9.1). Within these twelve localities, four were devoid of *D. japonicus*, in the northern side of *D. suweonensis* range, although sample sizes for these populations were the lowest ($3 < n < 13$), and the species was detected at these sites in subsequent surveys (Borzée et al. 2017b).

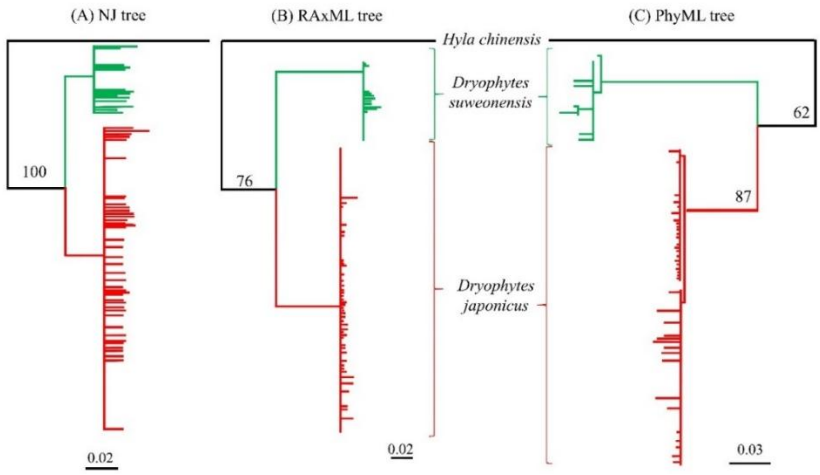


Figure 9.3. Phylogenetic trees inferred using (A) Neighbour Joining and Maximum Likelihood using (B) RAxML and (C) PhyML. Numbers on the major branches indicate bootstrap support >50. All trees are built with *Hyla chinensis* (GenBank accession number AY458593.1) as outgroup. All trees are congruent, with a subclade within the PhyML tree as the only difference. All trees were built in Geneious (v 9.04, Biomatters Limited, Auckland, New Zealand) with corresponding plug-ins, and all nodes below 30 % support were collapsed. Colours represent the two species.

Microsatellites analysis

Genotyping was successful for 381 individuals from 34 populations (Annex 9.3). The analysis for the potential presence of a null alleles highlighted a homozygote excess at RHO. However, due to the low frequency of the two most numerous classes of alleles, respectively 0.58 and 0.16 in five populations, we kept it for further analysis. Also, the analysis presented no evidence of scoring error due to stuttering and no evidence for large allele dropout. None of the loci was detected to be following HWE due to heterozygote deficit (SE = 0.001, Switches = 1589.2, $p > 0.001$).

Based on the species assignment through mtDNA and through F-statistics, we detected low gene diversity per locus when comparing expected and observed heterozygosity. The respective average values of 0.29 and 0.24 were found for populations harbouring *D. suweonensis* and *D. japonicus* only, while H_e at sites with both species was 0.41. Besides, the number of alleles per locus (A_e) for site with either species was low, but twice higher when both species were present. The patterns of allelic richness (AR) per locus were the same, with a mean of 1.28 alleles for sites with *D. suweonensis* only, 1.23 alleles for populations with *D. japonicus* only, and 1.40 for populations where both species were present. The degree of inbreeding (F_{IS}) was on average double for populations with *D. japonicus* only (0.61) than for populations with *D. suweonensis* only

(0.37), reflecting heterozygote deficiency. However, F_{IS} was not significantly different between averages for sites with one species compared to sites with both species ($T = 0.853$, $p = 0.433$).

The genetic differentiation between populations (F_{ST}) was 0.22 on average between populations of *D. suweonensis*, while a value of 1.19 between populations of *D. japonicus* represents higher gene exchange between populations. The AMOVA on F_{ST} values computed through IMa2 reported an estimated variation among populations of 0.45 (*df* = 3, *SS* = 332.20, *MS* = 14.44), an estimated variation among individuals of 0.67 (*df* = 319, *SS* = 546.04, *MS* = 1.72) and a variation within individuals of 0.37 (*df* = 343, *SS* = 128.5, *MS* = 0.375). The genetic variation among populations therefore accounts for approximately 30 % of the total genetic variation, while the variation between individuals is approximately 45 %. The second AMOVA between observed and random F_{ST}, based distance matrices showed a negative correlation between genetic and geographical distances (*Rxy* = -0.074, P(*rand> = data*) = 0.001). Finally, the analysis for bottlenecks detected bottlenecks for all *D. suweonensis* populations, and three *D. japonicus* populations.

The results of the analysis based on the pairwise F_{ST} values displayed a strong interspecies divergence (0.30, $p < 0.01$), but also the presence of two clades within *D. japonicus* (0.28, $p < 0.01$), without strong geographical representation. Following the same pattern, *D.*

suweonensis is also divided in two clades (0.35, $p < 0.01$), one north and one south of the city of Seoul and the Han River.

Population structure

The Bayesian analysis of population with STRUCTURE selected K = 2. The mean LnP(K) = 2786.41 for K = 1, mean LnP(K) = 3784.83 for K = 2 and mean LnP(K) = 3518.07 for K = 3. For K = 2, the mean value of F_{ST} for the two clades were respectively 0.09 and 0.35. Out of 381 individuals, and based on a 10 % minimal purity threshold, STRUCTURE assigned 124 individuals to *D. suweonensis*, 230 individuals to *D. japonicus* and 27 individuals were qualified of hybrids (Fig. 9.4A). No intraspecific substructure was detected within either "pure" populations, as the estimated Ln Probability of Data averaged over 10 runs was higher for "K = 1" within *D. suweonensis* (K = 1: 1409, K = 2: 1106, K =3: 1032) and within *D. japonicus* (K = 1: 2933, K = 2: 2318, K =3: 2156).

The STRUCTURE analysis for the West-East transect (n = 172) highlighted the absence of both "pure" *D. suweonensis* and hybrid individuals east of the start of the Baekdudaegan Mountain Range (Fig. 9.4B). All "pure" *D. suweonensis* and hybrids were located in lowlands (Fig. 9.4A). The STRUCTURE analysis for the North-South transect (n = 201) highlighted the presence of hybrid individuals and some "pure" *D. suweonensis* further south than the known range of the species based on

calls (Borzée et al. 2017b). Besides, the majority of hybrid individuals were south of the southern limit of *D. suweonensis* range (Fig. 9.4C). Finally, three hybrid individuals were unexpectedly found east of the Baekdudaegan Range in southern localities; two were clearly hybrids, but one was close to the 10 % assignment threshold. The furthermost individual was from the population in Uljin (assignment to *D. japonicus circa* 87 %), a second one was from the population in Kyeongju (assigned to *D. japonicus circa* 35 %) and the last one was from the population in Goseong (assigned to *D. japonicus circa* 48 %; Fig. 9.4).

Identification of hybrids

Comparing STRUCTURE analysis and mtDNA assignments, matching assignments were reported for 234 individuals for the *D. japonicus* clade, and 68 to the *D. suweonensis* clades. STRUCTURE-defined hybrid individuals were found at 15 sites (Fig. 9.4), for a total of 33 individuals, among which 22 were assigned to *D. japonicus* and 11 to *D. suweonensis* based on mtDNA. These individuals were assigned to the two clades at varying degrees of confidence, therefore suggesting admixture. The remaining 46 individuals exhibited cytonuclear disequilibrium, with eight individuals assigned to *D. japonicus* through mtDNA and *D. suweonensis* through microsatellites, and 38 individuals with the reverse situation. No geographic pattern for individuals in cytonuclear disequilibrium was detected.

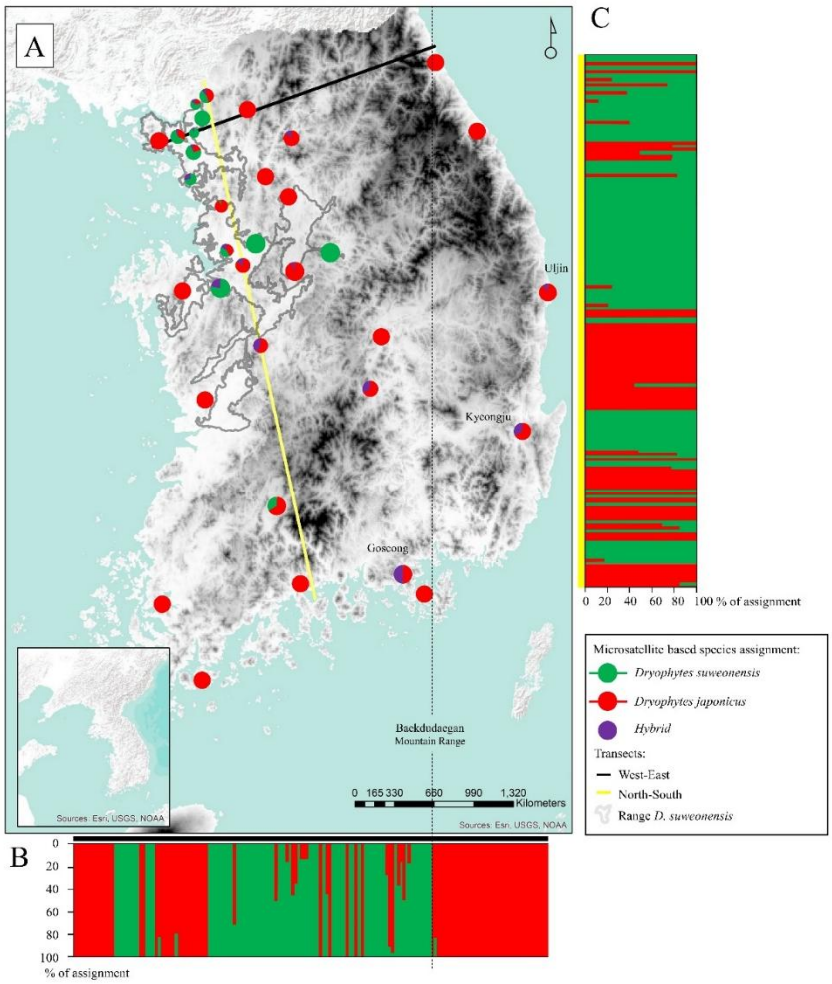


Figure 9.4. Structure analysis of microsatellite data, assigning each individual to a clade based on percentages of assignment. (A) pie charts represent the percentage of pure and hybrid individuals for each population, while the two transects (B – West-East; C North-South) represent individual assignments within populations. Structure assignments were drawn only if reaching the 10 % threshold. Service Layer Credits: Sources: Esri, USGS, and GeoServicesMap Esri Korea. Map generater in ArcMap 10.5 (Esri, Redlands, USA).

DISCUSSION

Our results demonstrated that widespread hybridisation and bi-directional introgression do occur, on the totality of the range of *Dryophytes suweonensis*. Besides, if the current trend persists, *D. suweonensis* is under risk of extinction because of hybridisation, as highlighted by the high number of individuals in cytonuclear disequilibrium (Jiggins and Mallet 2000; Allendorf et al. 2001).For a definitive demonstration of speciation reversal, it would be required for *D. suweonensis* to go extinct. This would *allegedly* lead the two *Dryophytes* species to "reverse" to the ancestral species (Seehausen 2006; Vonlanthen et al. 2012). It is thus impossible to say if speciation reversal will happen for *D. suweonensis* and *D. japonicus* without the loss of these evolutionary significant species (Dufresnes et al. 2015). Furthermore, F_{IS} was found not to be different between sites with either or both species, thus highlighting that hybridisation is one of the main problems for *D. suweonensis*, and not inbreeding, as commonly found in declining species (Frankham 1998; Spielman et al. 2004b; Frankham 2005).

When assessing the match between bio-geographical patterns and the clade partitioning and heterozygosity deficit in our results, the genetic structure within both species corresponds to the anthropomorphised landscape features. In the case of *D. suweonensis*, the city of Seoul creates a permeable barrier between northern and southern populations, with two

remote stepping stone populations (Borzée et al. 2015a), likely resulting in interrupted gene flow. For *D. japonicus*, the lack of sub-clade structure presented by the NJ and RAxML trees, in comparison to PhyML, is not expected to pertain to genetic variations, does not reflect geographical variations, and is likely the results of the less robust analysis (Yang and Rannala 2012). This is supported by K = 1 from the STRUCTURE analysis of mtDNA assigned *D. japonicus* individuals, and the lack of strong geographical structure in the two *D. japonicus* clades founds based on the F_{ST} matrix. We demonstrated the presence of bottlenecks in all *D. suweonensis* populations, which reflects the current situation with the lack of dispersion routes potentially leading to gene exchange, because of the fragmentation of the habitat (Borzée *Chapter 2*). The F_{ST} values for *D. suweonensis* were much lower than the average for amphibians (Grobler et al. 2003; Andersen et al. 2004; Arens et al. 2006; Angelone and Holderegger 2009), likely related to the bottlenecks, *i.e.* lower dispersal rate, fragmented habitat, and small population size (Freeland et al. 2011 Bilton et al. 2001; Park et al. 2013)

Both haplotypes networks displayed evidence of population expansion, matching with ecological data for both species. The population expansion is expected to have followed agricultural development for both species, but also recent expansion to sites of agriculture on reclaimed mudflats by *D. suweonensis* (Borzée et al. 2017b). The two haplotype

networks also revealed number of missing haplotypes. In the case of *D. suweonensis*, missing haplotypes are likely a direct effect of land transformation at the centre of the range of the species (Borzée et al. 2015a; Borzée et al. 2017d; Borzée *Chapter 2*). Accordingly, genetic relatedness was not correlated with distance between populations, as gene exchange cannot occur between populations, and thus populations that are one motorway apart are functionally as close as populations from different continents.

Although bi-directional, introgression seems to be relatively higher for individuals assigned to *D. suweonensis* through mtDNA (38 vs. eight individuals). This unbalanced ratio is likely related to mating behaviour of calling males. Male *D. suweonensis* call from the centre of rice paddies, while male *D. japonicus* call from the edges (Borzée et al. 2016a; Borzée et al. 2016b). As female *D. suweonensis* move toward the centre, they must cross a barricade of male *D. japonicus* in order to reach conspecific males. The same pattern has also been described in other treefrog species, such as *D. cinereus* and *D. gratiosus* (Lamb and Avise 1986). As male anurans are undiscerning about the species they mate with (Brown 1977; Reading 1984), hybridisation is likely when they grab non-conspecific females. In fact, male *D. japonicus* have been observed amplexed with several other species (*Rana amurensis*, *R. uenoi*, *Hynobius leechii*, *Pelophylax nigromaculatus*, *P. chosenicus*, author's personal

observations).

The presence of populations with hybrid individuals was geographically structured: all sites with hybrid individuals were situated on the periphery of *D. suweonensis*' range (Figs. 51 and 54). This corresponds to the hypothesis suggesting that *D. japonicus* was not distributed on lowlands before landscape anthropisation, and thus peripheral populations of *D. suweonensis* have been under exacerbated hybridisation pressure since large scale landscape modification following the Korean War (1950-1953). The presence of individuals in cytonuclear equilibrium outside of *D. suweonensis*' range, and further than the maximum dispersion distance for a single generation (Smith and Green 2005), also demonstrates that backcrossing of hybrid individuals into *D. japonicus* gene pool is not limited to the F1 generation. The North-South transect selected along the low plains of western Korea showed that at the contrary to the West-East gradient, the habitat is adequate for *D. suweonensis*, or at least to individuals resulting from hybridisation. It is however interesting to see that although hybrids are found further south than the range of *D. suweonensis*, no individual with the call properties of the species was found as far south, with the Mankyeong River acting as the southern limit of individuals with the call properties of *D. suweonensis* (Borzée et al. 2017b). Some hybrid individuals were found at southern localities east of the Baekdudaegan, but none were identified as *D.*

suweonensis based on mtDNA, thus suggesting repeated backcrossing south of mountains, over many generations. This hypothesis is supported by the increased contact between the two clades through agriculture (Kim 2016; Borzée *Chapter 8*), which started circa 5000 years BC (Fuller et al. 2008). The fact that no hybrid was found further east than the easternmost limit of *D. suweonensis* range at northern latitudes shows that the habitat is not adequate for the species (Roh et al. 2014), but also that hybrids inherit traits that prevent them from exploiting the habitat, at the contrary to pure *D. japonicus* individuals.

Chapter ten

A RING-SPECIES OR A RING OF SPECIES? PHYLOGENETIC RELATIONSHIP BETWEEN TWO TREEFROG SPECIES, *DRYOPHYTES SUWEONENSIS* AND *D. IMMACULATUS*, AROUND THE YELLOW SEA.

ABSTRACT

Phylogenetic patterns due to glaciations are still understudied in North East Asia (NEA). Besides, the effects of the Last Glaciation Maximum on phylogenetic patterns are less explicit in NEA than other regions of the northern hemisphere, due to topographically homogenous landscapes. Here, we aim at assessing the phylogenetic status of the *Dryophytes suweonensis* and *D. immaculatus* clades. We used concatenated partial mitochondrial 12S and 16S gene fragments, with a combined length of 678 bp for *D. suweonensis* (*n* = 32) and *D. immaculatus* (n = 5) collected from the Republic of Korea and the People's Republic of China, respectively. *Dryophytes suweonensis* formed an apparently monophyletic clade, whereas *D. immaculatus* did not. Our results also demonstrated the continuous genetic variation through haplotypes forming a ring around the Yellow Sea. It is therefore difficult to conclude on either a ring-species or a ring of species around the shallow Yellow Sea, which acted as a land-bridge several times during

recent geological times. We recommend the use of other cues such as call characteristics and morphology to determine the species or sub-species status of these two clades.

INTRODUCTION

The impact of glacial cycles on speciation events in Europe and North America has been clearly demonstrated for a number of species (Hewitt 2000; Knowles 2001; Veith et al. 2003; Avise 2007). For instance, many amphibian species, including the *Hyla arborea* complex, survived glaciation cycles through the use of peninsular refugium in Southern Europe (Stöck et al. 2008; Stöck et al. 2012). However, this kind of study is largely missing in North East Asia. The impermeable geographical barrier to gene flow is the Himalayan range and the adjacent southern edge of the Tibetan plateau. These topographical patterns delimit a southern edge of species distribution, as visible in *Bufo gargarizans* (Yan et al. 2013, Borzée et al.2017e). Glaciation cycles did influence speciation events for peninsular populations, as demonstrated in the split between *B. gargarizans* and *B. bankorensis* (Chen et al. 2013; Yu et al. 2014), and *Hyla chinensis* and *H. simplex* (Hua et al. 2009). Because of sea level rise and recession, the Korean peninsula was alternatively isolated from the Japanese Archipelago and connected to the Chinese mainland. During glacial oscillations, the peninsula became totally isolated and acted as an

isolated refugium during the last glacial maximum (LGM), even for good dispersers such as racoon dogs (Kim et al. 2013). The Korean Peninsula was not covered by glaciers, but it was colder and drier during glacial periods (Kong 2000; Yi and Kim 2010) and acted as one of the two refugia during the late Pleistocene, together with now central China. For instance, *Pelophylax nigromaculatus* (Zhang et al. 2008) and *Onychodactylus fischeri* (now assigned to *O. koreanus*; Yoshikawa et al. 2008) benefitted from these refugia. Besides, the Korean peninsula itself is divided by non-crossable landscape elements, leading to genetic diversification within *P. chosenicus* (Min et al. 2008), *Hynobius* spp. (Baek et al. 2011; Min et al. 2016), and *Dryophytes japonicus* (Dufresnes et al. 2016).

Glaciation cycles lead to species specific speciation scenarios, with clearly divergent species separated by the Yellow Sea, such as *P. chosenicus* in Korea and *P. plancyi* in China, (see Liu et al. 2010). However, the same conditions resulted in the uninterrupted gene exchange over very large geographic area, such as for *B. gargarizans* over the totality of North East Asia (Borzée et al. 2017b). Intermediate and non-resolved cases also exist, such as for the *D. suweonensis/immaculatus* complex (Li et al. 2015; Dufresnes et al. 2016, Borzée et al. 2017a). Besides, studies on the divergence date for these two species and the sister clade *D. japonicus* are not consistent. The divergence with *D.*

immaculatus is dated to 14 mya (Hill 2009) while the divergence with *D. suweonensis* is dated between 6.4 and 5.1 mya (Li et al. 2015; Dufresnes et al. 2016).

The origin of the *Hyla* genus (Smith et al. 2005; Faivovich et al. 2005 and Hua et al. 2009; Wiens et al. 2005; Wiens et al. 2010) is consistent with Savage (1973) hypothesis of a late Cenozoic invasion. The northern oriental *Hyla* populations belong to two main groups, namely "*H. arborea* group" (*sensu stricto* Anderson and Green (1991) and *"D. japonicus* group" (*sensu stricto* Hua et al. (2009). The genus Hyla was recently renamed as *Dryophytes* (Duellman et al. 2016). The former originated from Northern America through the Bering pass (Anderson and Green 1991; Borkin 1999; Duellman 2001) 28 to 23 mya (Smith et al. 2005) and diverged between 22 to 18 mya into the Asian and European clades (Riehl et al. 1995; Smith et al. 2005). The "*D. japonicus* group", comprising *D. japonicus*, *D. stepheni* (Dufresnes et al. 2016), *D. suweonensis* (Kuramoto 1984; Riehl et al. 1995) and *D. immaculatus* (Hua et al. 2009), reached Asia during a second expansion by the "*D. eximia* taxa" (Anderson and Green 1991; Borkin 1999; Faivovich et al. 2005) from the American continent (presence of a common Nuclear Organizer Region in chromosome 6; Hua et al. 2009) between 18.9 and 18.1 mya (Smith et al. 2005).

The *Dryophytes* and *Hyla* Asian genera are largely parapatric

(Hua et al. 2009), with an area of overlap from 28 to 33° N latitude (Hoffmann 2001, Hua et al. 2009). This may lead to potential competition, such as between *H. chinensis*, from the "*H. arborea* group", occurring together with *D. immaculatus*, from the *"D. japonicus* group*"* (Anderson and Green 1991; Hua et al. 2009). Other ambiguities arise, such as the relatedness of calls between *D. japonicus* and *H. hallowelli* (Kuramoto 1980), although they do not belong to the same group. On the other hand, *D. japonicus* (potentially *D. stepheni*, see Dufresnes et al. 2016) and *D. suweonensis* calls are distinct, despite their phylogenetic promiscuity (Riehl et al. 1995, Faivovich et al. 2005; Hua et al. 2009). Finally, the divergence between treefrogs of the "*D. japonicus* group" started 24 mya (Riehl et al. 1995), and supposedly divided into *D. japonicus* and *D. immaculatus* 14 mya (Hill 2009), while *D. japonicus* and *D. suweonensis* diverged between 6.4 and 5.1 mya (Li et al. 2015; Dufresnes et al. 2016). This lack of congruence may be explained by the prior split of *D. japonicus* and the mainland clade, potentially *D. stepheni* (see Dufresnes et al. 2016).

Since Gunther (1958), and Kuramoto (1980), two clades of treefrogs have been identified on the Korean peninsula: *D. japonicus* and *D. suweonensis* (Yang and Park 1988b; Yang et al. 1997; Lee et al. 1999; Duellman et al. 2016, Dufresnes et al. 2016). On the opposite side of the Yellow Sea, the Chinese mainland is populated by *D. japonicus* and *D. immaculatus*. Here,

we hypothesize that the *D. suweonensis/immaculatus* complex is composed of two divergent species when taken at the extremities of the ranges, but less clearly differentiated when in proximity, and thus form a ring species.

MATERIAL AND METHODS

Species description

Dryophytes suweonensis populations have been recorded as declining throughout the last decade (IUCN 2017a) and are today restricted to a narrow land strip between the Yellow Sea and the western edge of the Baekdudae Range (Roh et al. 2014; Borzée et al. 2016c). This spatial location and the urban development lead to the isolation of populations and poses a threat to its survival in the long term.

Field sampling

Field sampling was conducted over three localities, encompassing the totality of the known range of *D. suweonensis* (Borzée and Jang 2015; Borzée et al. 2016c). This was necessary as *D. suweonensis* populations are fragmented due to landscape barriers such as Metropolitan Seoul and the Geum River (Borzée et al. 2015a). The three localities were Geumchon (North of Seoul, n = 12, annotated as BHV on subsequent tables and figures), Cheonan (South of Seoul, n = 10, annotated SUR), and Iksan, a non-connected population on the Southern edge of the species'

range (n = 10, annotated LTR). A distance of at least 90 km separated the adjacent localities. The samples at the two northern localities were collected in 2013 (Annex 10.1) under the Ministerial authorisation number 2013-16, while the Iksan locality was sampled in 2014 under the permits 2014-04, 2014-08 and 2014-20. Due to the endangered status of the species, we followed the strict ethical regulation dictated in the permits.

Each locality was sampled once only to prevent pseudo-replication (Annex 10.1). Localities were presumed to be independent of each other, as the dispersion range of most amphibian species is below 10 km per year (Smith and Green 2005), and that of hylids not over more than a couple kilometres per season (Vos et al. 2000; Smith and Green 2005; Arens et al. 2006). Genetic material was acquired through oral (= buccal) swabs (Broquet et al. 2007), frozen within 24 h at -20°C.

Molecular work

Mitochondrial DNA was subsequently extracted with the Enzynomic® Genomic DNA Extraction Kit (Tissue; Genomics Extraction Kit protocol V2013-1; Seoul, Korea) for Geumchon and Cheonan samples and the Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany) for Iksan samples, following the instructions of the manufacturer.

Because *D. suweonensis* and *D. japonicus* are difficult to identify based on morphology only (Borzée et al. 2013), each individual was

identified as belonging to *D. suweonensis* based on mtDNA COI sequencing, following the protocol developed by Jang et al. (2011). This sequences were not used for further analysis as not sequenced for *D. immaculatus*. Then, as sequences of *D. immaculatus* were available from GenBank for partial mitochondrial ribosomal 12S and 16S genes (accession numbers KP742584 through KP742589 for 12S, and KP742712 through KP742716 for 16S, Annex 10.1), we sequenced all individuals for part of these genes. For 12S, new primers were designed with Geneious v.9.1.6 (http://www.geneious.com, Kearse et al. 2012; plug-in Primer3 v.2.3.4) from *D. immaculatus* sequences from GenBank: DIS-12S-F: CCC AAG ACA CCT AGC TAC GC; and reverse DIS-12S-R: TGG CTT TGA AGA GGG TGA CG. For 16S, the primers used were: 16SA-L CGC CTG TTT ATC AAA AAC AT and 16SB-H CCG GTC TGA ACT CAG ATC ACG T (Vences et al. 2005; Jeong et al. 2013), completed by specifically designed primers overlapping with the 16SA and 16SB primer set, designed such as above: DIS-16S-F: GTA AGG GCC CCA ACG TAG TC and DIS-16S-R: AGG GAT GCT GTA GTT AGG GGT.

PCR reactions using published primers were run following the protocols by Jeong et al. (2013). All newly designed primers were used in PCRs with each reaction containing 14.5 μL of distilled water, 2.0 μL of (10x) buffer, 1.6 μL of dNTPs (final concentration of 0.07 mM), 0.4 μL of each primer, 0.1 μL of Takara Taq polymerase and 1 μL of DNA. The

thermocycler (SimpliAmp Thermal Cycler; Applied Biosystems by Life Technologies; Carlsbad, California, United States) was programmed at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, with a terminal elongation at 72 °C for 5 min (12S) and 10 min (16S). Samples were then run on a 1.5 % agarose gel during a 12 min electrophoresis, and pre-stained with MaestroSafe dye (Maestrogen; Las Vegas, Nevada, USA). Samples were subsequently cleaned-up with the PCR Clean-Up Kit (LaboPass PCR, PCR Purification Kit; Cat. No. CMR0112; Cosmogenetech; Seoul, Republic of Korea).

Molecular analysis

The partially sequenced mitochondrial 12S and 16S ribosomal RNA genes, 628 bp and 485 bp, respectively, were proofread, edited, assembled, and concatenated with Geneious 9.1.6 (v9.04, Biomatters Limited, Auckland, New Zealand). Despite the 16S sequences being originally 485 bp, only 50 bp were used in the analysis, due to short overlap with the selected reference sequences for *D. immaculatus*. Details on specimen vouchers, GenBank accession codes, and sampling sites of the all sequences used for this study are listed in Annex 10.1. The alignment was performed using MUSCLE (Edgar 2004), implemented through the Geneious plug-in, with a maximum of 10 iterations following default parameters, and further revised manually when needed. The final alignment had a sequence length of 677 bp.

Estimation of genetic variables

We computed the haplotype diversity, Fu's F (Fu 1997) and Tajima's D test for neutrality (Tajima 1989) to infer whether the genes chosen, although slow evolving, were under selection or at mutation-drift equilibrium. The software DnaSP was used to estimate these three indices (Librado and Rozas 2009).

In order to refine the relationships between and within the two clades, we created a haplotype network in TCS (Clement et al. 2000) with a fix connection limit at 500 steps (estimated) for the concatenated partial 12S and 16S, with all other parameters set as defaults (Clement et al., 2000). PopART (Population Analysis with Reticulate Trees; http://popart.otago.ac.nz) was used to visualise the haplotype network.

Phylogenetic tree

Three types of phylogenetic trees were constructed to resolve the phylogenetic relationship between the two clades. We first employed jModelTest 2.1.10 (Darriba et al. 2012; Guindon and Gascuel 2003a) to estimate the best-fitting substitution model of the concatenated 12S and 16S rRNA genes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). We chose the General Time Reversible model with the addition of invariant sites and gamma distribution of rates across sites (GTR+I+G) for the reconstruction of the phylogeny (AIC data not shown).

We then created a PhyML tree with the PHYML plugin in Geneious (Guindon and Gascuel 2003a), as it implemented a fast and accurate heuristic for estimating maximum likelihood phylogenies. We applied the GTR substitution model with 50,000 bootstraps and all other variables as default.

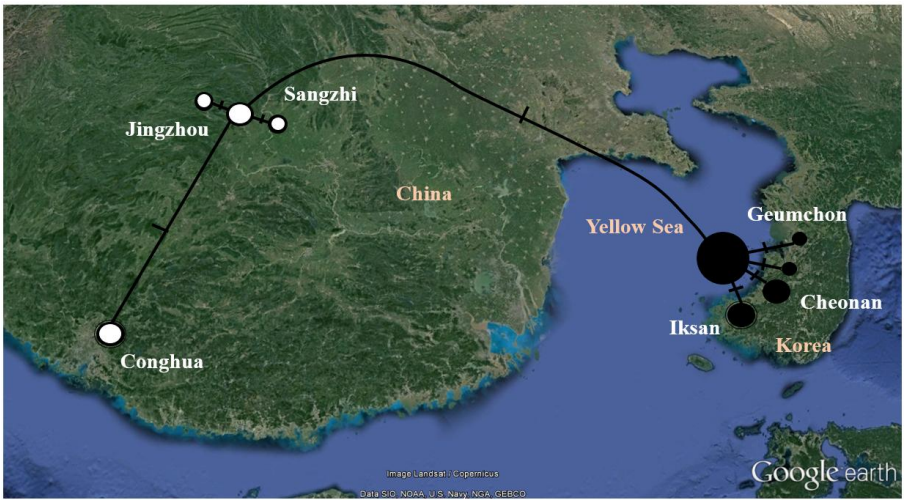


Figure 10.1. Haplotype network constructed through PopART overlapped on sampling localities for *Dryophytes immaculatus* (open circles) and *D. suweonensis* (solid circles). The population-specific haplotype found in Geumchon, north of Seoul for *D. suweonensis*, and Conghua, southernmost locality in China for *D. immaculatus*, are circled. The size of markers is representative of sample size ($1 \leq n \leq 22$). This figure was generated with ArcMap 9.3 (Environmental Systems Resource Institute, Redlands, California, USA) and Google Earth Pro (Mountain View, California, USA).

We also added *Pseudacris nigrita* and *Smilica fodiens* as outgroups to the analysis (GenBank accession number in Annex 10.1), such as suggested by Li et al. (2015).

We then constructed two phylogenetic trees to clarify the relationship between the two species. We first ran a RAxML tree (Geneious plugin; RAxML 7.2.8) with the model selected with 50 000 bootstrap replicates and all other variables set as default. *Pseudacris nigrita* and *S. fodiens* were also integrated as outgroups to the analysis. We finally ran a Bayesian Inference phylogenetic analysis with a single Markov Chain Monte Carlo (MCMC) analysis of 1.1 million iterations, each with 4 heated chains on Mrbayes 3.2.6 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). One tree was saved every 200 generations, and prior distribution default parameters were used. The final 50% majority rule consensus tree was saved. For this models, 18 species extracted from by Li et al. (2015) were used as outgroups, and we added four *Dryophytes japonicus* individuals to the analysis (all GenBank accession numbers in Annex 10.1).

RESULTS

Our results highlight the divergence, albeit potentially recent, between the two clades. *Dryophytes suweonensis* formed a monophyletic clade in both Maximum Likelihood and Bayesian Inference phylogenies.

The absence of shared haplotypes at the extremes of the ranges for both species implies the two species are distinct in terms of sequence structure for the concatenated 12S and 16S rRNA.

Estimation of genetic variables

The haplotype diversity calculated through DnaSP for *D. suweonensis* showed a low genetic variability with only five haplotypes (*Hd* = 0.54, variance = 0.00812). Fu's F (F = -1.15, $p > 0.10$) and Tajima's D test for neutrality (D = - 0.99, $p > 0.10$) were not significant, supporting no clear pattern of demographic dynamics. The conflicting values of Fu's Fu and Tajima's D may indicate a low sample size or recent population reduction or bottleneck. The haplotype network created (Fig. 10.1) highlights the non-overlap of common haplotypes between the two species, but also the same degree of variation (one nucleotide difference) between and among species. In addition, one of the *D. suweonensis* haplotypes is only found at the locality North of Seoul, while the isolated locality in Iksan does not display unique haplotypes. We found the same pattern for the southernmost locality for *D. immaculatus*, with all individuals from Conghua displaying the same haplotype (Fig. 10.1).

Phylogenetic analysis

The three models used with the phylogenetic analysis of the two concatenated mitochondrial genes were congruent for the monophyly of

D. suweonensis, although variations were visible within the *D. immaculatus* clade, and the branching of the outgroups. The ML tree constructed through PhyML highlighted the segregation between the two clades (Fig. 10.2), but also supported the non-monophyly of *D. immaculatus*. In contrast, the ML tree constructed through RAxML suggested the early divergence between the two species, while both species were presented as monophyletic (Fig. 10.3). The final Bayesian Inference analysis is congruent with both analyses on the monophyly of *D. suweonensis*, despite a posterior support of 0.512, and the phylogenetic relationships reflected by the RAxML analysis (Fig. 10.4).

DISCUSSION

The results of our phylogenetic analyses are congruent in the monophyly of the *Dryophytes suweonensis* clade. However, the haplotype network clearly shows the relatedness of *D. suweonensis* and *D. immaculatus* clades, and the same genetic variation between and within clades. This pattern could very well be the one of a ring species, although more variable markers such as microsatellites would be required to confirm such a claim.

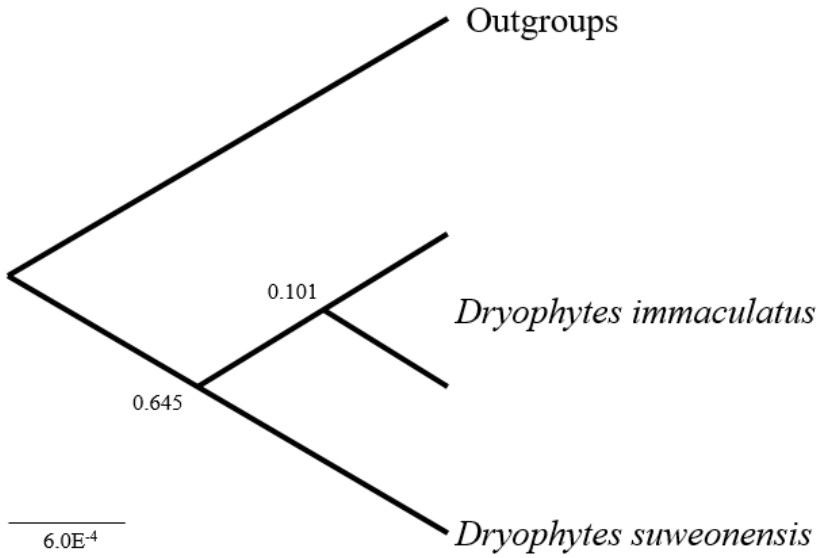


Figure 10.2. Simplified Maximum Likelihood Phylogenetic tree built with the PHYML plugin in Geneious. This analysis highlights the segregation between the two *Dryophytes* clades, and supports the monophyly of *D. suweonensis*.

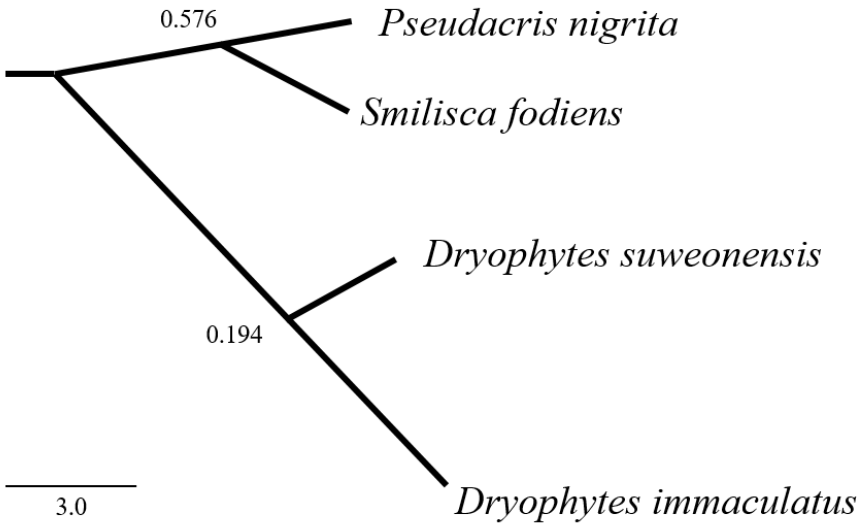


Figure 10.3. Simplified Maximum Likelihood Phylogenetic tree built with the RaxML plugin in Geneious. Here, both species were presented as monophyletic.

This pattern is consistent with the fact that landscape features usually are adequate predictors to genetic variations (reviewed by Storfer et al. 2007), such as seen here with the Yellow Sea creating a barrier between the two clades. Li et al. (2015) and Dufresnes et al. (2016) recommended the synonymy of *D. suweonensis* (Kuramoto 1980) and *D. immaculatus* (Boettger 1888), under the name *D. immaculatus* as junior synonym. We, however, call for further analysis before this conclusion.

To better determine the status of species or subspecies of the two populations, we recommend a Total Evidence Analysis that would include call properties, morphometrics and genetic information, such as conducted for the phylogeny of *Dendropsophus elegans* (Forti et al. 2017). It has been suggested that intra-specific variation in call properties are not adequate to recover phylogenetic history due to selective factors related to sexual selection. However, inter-species phylogeny is accurately described by acoustic properties due to genetic isolation processes, in relation to species recognition and ecological pressure (Fonseca et al. 2008; Forti et al. 2017).

Call variables are expected to be significantly different for species diverged for at least 6.5 million years (Forti et al. 2017). The alternation between periods of divergence and contact between *D. suweonensis* and *D. immaculatus* may have occurred repeatedly since the Triassic (Haq et al. 1987), with the repeated resurgence and decline of the Yellow Sea

(Jingtai and Pinxian 1980; Oba et al. 1991; Liu et al. 2009).

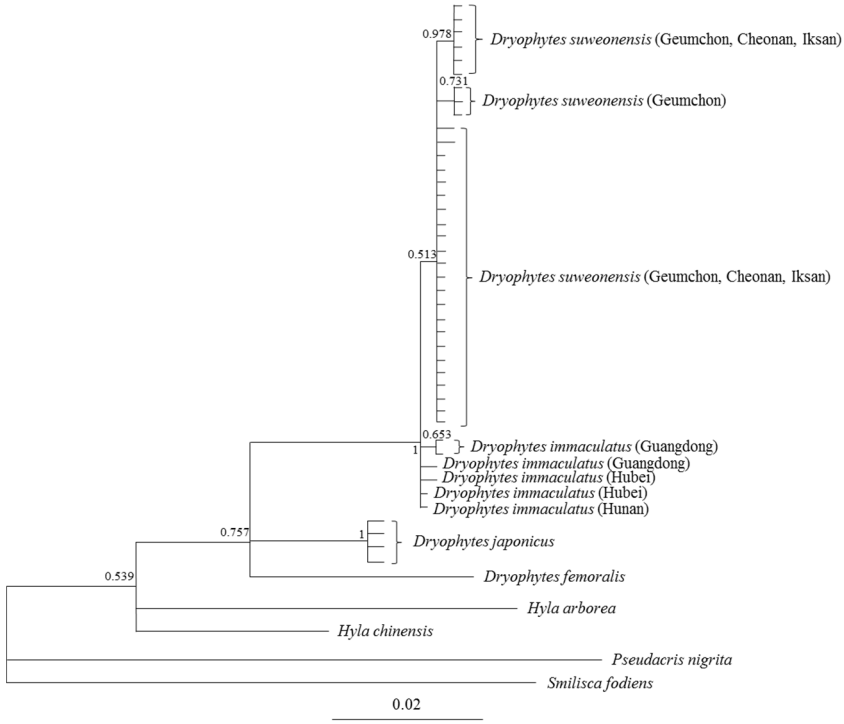


Figure 10.4. Bayesian Inference phylogenetic tree with a single Markov Chain Monte Carlo. This analysis also highlights the monophyly of *D. suweonensis*, despite some isolated branches.

Accordingly, the two clades may have been partially isolated, starting the process of speciation through isolation. Then, they were brought back in contact during glacial maxima and the creation of the land-bridge over the Yellow Sea, before being isolated again. This is supported by the fact that the Korean Peninsula is known to have acted as a refugium for the ancestor of *Pelophylax* and *Onychodactylus* species (Zhang et al. 2008; Yoshikawa et al. 2008), and thus enabled the survival of clades that had previously colonised the area.

Alternatively, the two clades may be part of a ring-species as individuals were not isolated in different refugia during LGM. This proposition is supported by the absence of ice sheets on the Korean peninsula circa 126,000 years ago (Kong 2000; Walker et al. 2009; Yi and Kim 2010), partially due to the presence of a shared monsoon regime because of the uplifting of the Himalayan range (An et al. 2001 ; Harris 2006).This is also supported by the fact that Korean peninsula was last connected to the Chinese mainland during the late Pleistocene (Millien□ Parra and Jaeger 1999). In this case, the clustering of individuals in a single clade implies the repeated gene flow between the Korean Peninsula and the Asian mainland. Besides, such clustering is supported by the shared paleo-river basin due to the southward flow of the Amur River, west of the Sakhalin Islands, due to the ice sheet blocking its current bed (Grosswald and Hughes 2005). Furthermore, the paeleo-Yangtzae and

paleo-Yellow rivers were closer than they are today and merged with the paleo-Han river (Ryu et al. 2008).

Within each of the Korean Peninsula and China, the North/South genetic structure was unexpected because of the continuous low plains allowing for easy dispersion of individuals, such as exemplified by *D. suweonensis* (Borzée et al. 2015b). This may indicate strong philopatry of *D. suweonensis*, leading to a larger genetic divergence than expected and population management for *Dryophytes spp.* needs to take into account the origin of individuals for introduction and translocations plans (Gascon 2007). This is especially true if the individual *D. suweonensis* isolated on the phylogenetic tree Fig. 10.4 are not monophyletic with the main *D. suweonensis* clade, due to hybridisation with *D. japonicus* (Borzée et al. 2015b).

Appendix seven

COMPLETE MITOCHONDRIAL GENOME OF *DRYOPHYTES SUWEONENSIS* (ANURA HYLIDAE).

ABSTRACT

Dryophytes suweonensis is an endangered species with fragmented and declining populations from the Korean peninsula. We described 17,448 bp of *D. suweonensis* mtDNA, which had a shorter D-loop than other closely related species. The variation in nucleotide composition was similar to that of *Hyla tsinlingensis* but was larger than the one of its sister clade, *D. japonicus*.

BACKGROUND AND ANALYSIS

Comparative genetic analyses between the widespread *Dryophytes japonicus* and the endangered *D. suweonensis* are scarce, only specifying that the two species diverged about 5 mya (Li et al. 2015). Variations in behavioral ecology are now better understood, with clear behavioral and ecological differentiations between the two species (Jang et al. 2011; Borzée et al. 2016b). Sequencing the full mtDNA loop of *D. suweonensis* will enable the future development of primers to help identify populations of importance for conservation.

One *D. suweonensis* was caught in Pyeongtaek, Republic of Korea (37.001°N; 127.0055°E) in June 2015 for buccal swabbing. DNA was extracted (Qiagen DNeasy, Hilden, Germany) following the instructions of the manufacturer. Primers for two Long and Accurate (LA) PCRs were designed based on *Hyla tsinlingensis* mtDNA (GenBank accession KP212702), with *ad hoc* primers for the D-loop due to size difference with other hylids (Geneious v. R 9; Auckland, New Zealand). Sanger sequencing was conducted by Cosmogenetech (Seoul, Republic of Korea), before GenBank upload under accession number KX854020.

The nucleotide composition for *D. suweonensis* was 29.3% A, 27.2% C, 15.0% G, and 28.6% T. The total nucleotide composition was 17,448 bp long, thus 847 bp shorter than *Hyla tsinlingensis,* with only 0.1 % variation in the C nucleotide frequency (Huang et al. 2014). However, when compared with *D. japonicus* (accession number IABHU6123), it was 2,071 bp shorter, with a variation in nucleotide frequency of 0.3 % A, 1.8 % C, 0.5 % G and 1.9 % T. Using the Geneious Tree Builder plugin on Geneious v. R 9, a Neighbor-Joining Tree was constructed under the Tamura-Nei model and a 65% (5/-4) cost matrix, based on the complete mitogenetic sequences of all 11 *Hyla* uploaded on GenBank (Fig. A7.1), and with *Bombina orientalis* as outgroup.

The grouping of *D. suweonensis* and *H. tsinlingensis* together,

while another *H. tsinlingensis* is clustered within the *Hyla* clade, indicates a potential miss-identification of the two *H. tsinlingensis* individuals. An alternative identification would be *D. immaculatus*, due to the close relatedness with *D. suweonensis* (Li et al. 2015), and thus highlighting the divergence between these two species.

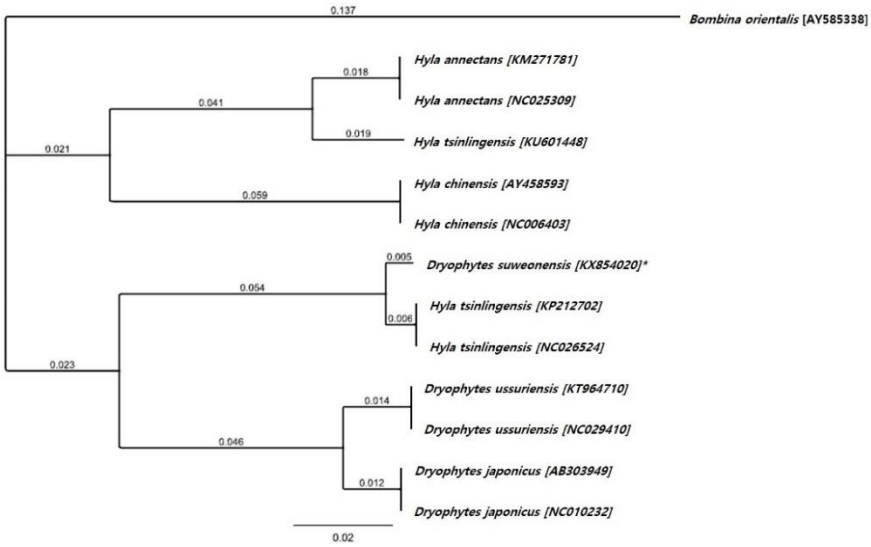


Figure A7.1. Neighbor-Joining Tree using all complete mitogene sequences available for the sister-genera *Hyla* and *Dryophytes*, with *Bombina orientalis* as an outgroup. The asterisk indicate the individual sampled in this study. Branch labels are substitutions per site.

PART IV: OTHER FACTORS OF POPULATION DECLINE AND CONSERVATION ASSESSMENT

This part contains all non-covered subject linked to the endangered status of amphibians. Here, this includes the relationship with pollutants (chapter 11), the potential for translocation (chapter 12) and population viability analyses (chapter 13).

Chapter eleven

IMPACT OF WATER QUALITY ON THE OCCURRENCE OF TWO ENDANGERED KOREAN ANURANS: *DRYOPHYTES SUWEONENSIS* AND *PELOPHYLAX CHOSENICUS.*

ABSTRACT

Water contaminants play an important role in threatening amphibian species already in the midst of the sixth extinction. Species relying exclusively on agricultural habitats for breeding are under multiple, and sometimes synergistic, threats because of the high levels of contamination from chemicals used as plant fertilizers and pest control. In the Republic of Korea, two anuran species listed as endangered occur primarily in rice paddies: Suweon Treefrogs (*Dryophytes suweonensis*) and Gold-spotted Pond Frogs (*Pelophylax chosenicus*). The purpose of this study was to describe the relationship between the occurrence of these two species and water contaminants in rice paddies. Water quality and contaminants were assessed through multi–site triplicate sampling. The rice paddies where the two anuran species occurred were characterized by higher concentrations of iron and lower concentrations of phosphate than rice paddies without the two species. Furthermore, low conductivity in the water was correlated to the occurrence of *P. chosenicus*, and low ammonium nitrate was a reliable predictor for the occurrence of *D.*

suweonensis. These contaminants principally result from the application of chemical treatments on agricultural fields. The decline of these two endangered anuran species might be related to current agricultural practices.

INTRODUCTION

Amphibian species are among many organismal forms faced with risks of extinction (Stuart et al. 2004). This unfortunate distinction stems from their sensitivity to a large number of factors (reviewed in Beebee and Griffiths 2005). Anthropogenic alteration of the landscape and emerging pathogens, especially chytrid fungus, are among the principal factors of population declines in many amphibian species (Skerratt et al. 2007; Kato et al. 2010; Moreira and Maltchik 2015). Other factors, such as environmental contamination, also negatively impact amphibian populations (Peltzer et al. 2006; Relyea and Hoverman 2006; Marco and Ortiz-Santaliestra 2009; Kerby et al. 2010; Egea-Serrano et al. 2012; Peltzer et al. 2013; Sasaki et al. 2015). The varied effects of contaminants on amphibians (reviewed in Sparling et al. 2010) range from those of pesticides on *Rana muscosa* (Davidson and Knapp 2007), to the correlation between pollutants and the decline of *Acris crepitans* (Reeder et al. 2005) and *Desmognathus fuscus* (Bank et al. 2006).

Species in anthropologically modified environments are typically exposed to synergistic threats that include exposure to contaminants, for which toxicity is thus difficult to assess in the field. Toxicity assessments in laboratory experiments might be inadequate representations of natural systems because of the complex additive effects of chemicals (Sparling et al. 2010). For instance, experiments on *Lithobates clamitans* showed that the application of both nitrate and carbaryl, present together in natural environments, resulted in decreased developmental rate and mass at metamorphosis, while the application of only one of the two chemicals had the opposite effect (Boone et al. 2005). Similarly, the combined effects of nine chemicals were stronger than that of one alone, and affected both growth rate and development of the endocrine system in anurans (Hayes 2005). The effects of contaminants are also impacted by physical properties of the environment, such as temperature (Boone and Bridges 1999; Broomhall 2004).

Besides direct negative effects on development, some contaminants also have an indirect negative effect (Sparling et al. 2010; Peltzer et al. 2013). For instance, both Roundup, the most commonly used glyphosate herbicide in the world, and carbaryl are known to decrease the abilities of several amphibian species to detect predators (Relyea 2003, 2005). Other contaminants negatively impact amphibian survival, and ultimately fitness, through depression of the immune system (Christin et

al. 2003; Linzey et al. 2003; Houck and Sessions 2006). Finally, when pollutants are found in agriculturally modified landscapes, they can have a negative impact on growth and development (Peltzer et al. 2013).

Those amphibian species that breed in agricultural landscapes (e.g., rice paddies) are often resorting to this habitat because they lack access to naturally-formed wetlands (Fujioka and Lane 1997; Elphick 2000; Lawler 2001; Magle et al. 2012; Naito et al. 2013). For example, species breeding in wetlands modified for rice production (*Rhacophorus ornativentris* and *R. arboreus*) exhibited differing patterns of abundance as a function of the availability of adjacent forest cover, while the abundance of both species was higher at intermediate water depths (Kato et al. 2010). This is also true in tropical landscapes, where, for instance, anuran diversity at agricultural ponds in Argentina is related to the reduction in pond size and depth, vegetation richness, and width of hedges (Peltzer et al. 2006). Amphibians breeding in wetlands that are used for cultivation, exposure to pollutants typically includes those originating from fertilizers, pesticides and heavy metals (Moreira and Maltchik 2014; Sasaki et al. 2015). These pollutants are introduced through direct application, run-off, sewage and atmospheric deposition (reviewed in Egea-Serrano et al. 2012).

Despite the relative ease of transforming natural wetlands to rice paddies (Czech and Parsons 2002), areas that are intensively cultivated for

rice production sometimes have high species diversity compared to adjacent habitats (Elphick and Oring 1998; Getzner 2002; Holzer 2014; Fasola and Ruiz 1996; Machado and Maltchik 2010). Moreover, management practices associated with rice production can have various impacts on the amphibian populations that breed in those habitats. As examples, flooding during the fallow phase in Brazil was not associated with amphibian richness (Machado and Maltchik 2010), while it appears to promote diversity in California rice fields (Brouder and Hill 1995; Elphick and Oring 2003).

As of 2003, ~89% of the world's rice paddies were distributed in Asia (FAO Stat 2004). In the Republic of Korea, 90% of the arable lands are used for rice production (U.S. Library of Congress 2015), and natural wetlands are very rare, especially in lowland regions. Two frog species occurring in that country, Gold-spotted Pond Frogs (Ranidae: *Pelophylax chosenicus*) and Suweon Treefrogs (Hylidae: *Dryophytes* [*Hyla*] *suweonensis*; Duellman et al. 2016), breed principally in rice paddies or their adjacent ditch network (Ra et al. 2008; Jang et al. 2011; Borzée and Jang 2015; Borzée et al. 2015). Both species are listed as vulnerable or endangered (IUCN 2016), but the reasons for their rarity are not fully known. Water contamination at the breeding sites is a potential contributor because there are several agricultural chemicals that are not currently regulated by the Korean government (Park et al. 2014). In this study, we

assessed the occurrence of both anuran species in relation to environmental water properties and contamination by pollutants including nitrate, phosphate, and heavy metals.

MATERIAL AND METHODS

Study Species

The two focal species of this study occur in the low plains of the western region of the Republic of Korea (Borzée et al. 2016a, 2017a). Since the loss of the last natural site in 2015 (Borzée and Jang 2015), *Dryophytes suweonensis* occurs only in rice paddies, where males produce advertisement calls while perched on rice seedlings (Borzée et al. 2016b). *Pelophylax chosenicus* are present in rice-paddy complexes, but the species also occurs in waterways near rice paddies (Eom et al. 2007; Ra et al. 2008). Both species are syntopic with larger and more abundant sister taxa, *Dryophytes* [*Hyla*] *japonicus* and *Pelophylax nigromaculatus* (Borzée et al. 2013; Roh et al. 2014). All sites included in our study were modified for rice production after the Korean War (1950–1953) and we assumed that the variation between sites was negligible and did not attempt to quantify variables at the landscape scale. Furthermore, all sites were sited in wetlands oriented perpendicularly to water channels that were connected to a central ditch along what was originally the center of a valley. As such, all paddies were flooded through the influence of gravity

only.

Survey modalities

All field surveys for the detection of the focal species occurred prior to water quality assessment. The rice-paddy complexes where we assessed frog presence and water quality were clusters of up to several hundred rice paddies, interconnected by waterways. Our choice of particular sites within a given complex was based on criteria established for the presence of the two focal species (for *D. suweonensis*, Roh et al. 2014; Borzée et al. 2017a for *P. chosenicus,* Ra et al. 2008, 2010; Park and Sung 2009). We selected 40 sites with the intent of achieving a balanced ratio of sites where both species were present, those where only one species was present, and those where they were both absent. All sites were separated by more than the mean annual dispersal distance of amphibians (Smith and Green 2005), and located in different rice-paddy complexes > 2 km away from one another (Fig. 11.1). As such, we assumed that all sites were spatially independent.

The second criterion used to select the 40 sites was the absence of recent anthropogenic modifications to the landscape. All rice paddy complexes were sited < 100 m above sea level and were of modern design, with a central ditch did not follow natural topographic features. Additionally, because *D. suweonensis* has precise needs for water quality (Roh et al. 2014), all sites were used exclusively for rice production,

without other crops in adjacent wetlands.

Surveys for frog' presence

Species presence at each site was assessed using triplicate aural surveys on the following dates: 9, 10, 16, 23, 24 and 30 May, and 6, 7, 13, 20 and 21 June 2015. Call surveys were conducted after sunset and before 0200 h, matching with the calling activity of the species (Borzée et al. 2016c). Additionally, we visually surveyed for adult *P. chosenicus* during each assessment of water quality. During the study period, males of the two species produced advertisement calls and recent metamorphs were observed at some of the sites, which we used as confirmation of having selected appropriate dates for assessing the occurrence of the focal species. The survey protocol consisted of a 10-min aural survey along a transect line marked by the longest straight road crossing the rice-paddy complex.

Sampling water quality

After aural surveys, we assessed water quality at the same sites. Water samples were collected at the downstream end of each transect to ensure that each sampling was representative of the water quality within each rice-paddy complex. Samples at all 40 sites were collected on the same day to minimize the influence that changes in environmental conditions might have on water quality. On alternate sampling days, we reversed the order in which we collected samples from each of the sites so

as to minimize any bias attributable to fluctuations in daily temperatures. Sampling bouts were conducted at two-week intervals on 17 and 31 May, and 14 June 2015.

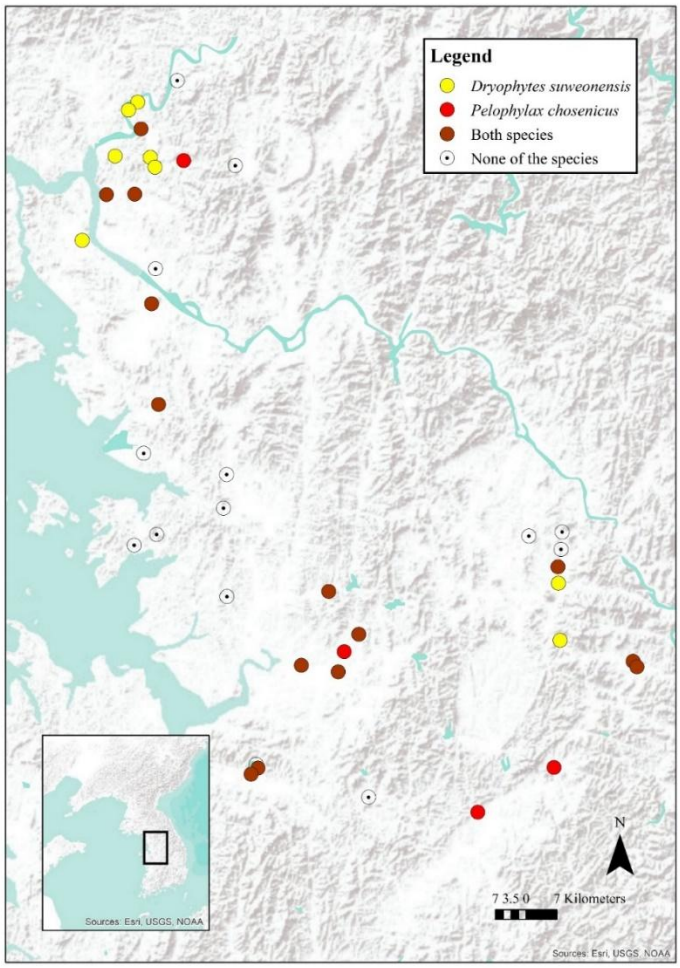


Figure 11.1. Sampling localities for the impact of water quality on the occurrence of two Korean anurans. *Dryophytes suweonensis* was present at 22 sites and *Pelophylax chosenicus* at 18. The two species overlapped at 14 sites. However, there were eight sites with *D. suweonensis* only and four sites with *P. chosenicus* only. All sites were located in different rice-paddy complexes ($n = 40$), which were at least 2 km away from each other.

The spatial arrangement of a typical rice-paddy complex allows for this protocol because a one-lane road traverses the middle of the complex parallel to the central ditch that irrigates it (detailed in Borzée et al. 2015, 2017a). The species were considered present if advertisement calls were heard during at least one of the three surveys.

For each site, we recorded temperature (°C), pH, conductivity (Siemens, S) and dissolved oxygen using a multi-parameter water quality sonde (600XL V2; YSI Inc.). The buffering capacity (KH; through calcium carbonate [$CaCO_3$]) and phosphate (PO_4) were tested with PMO test kits (Polystar Co., Ltd.) using the water volume required directly at the site. The range of KH measurements (0 to 450 mg/L) was divided into 25 intervals, representative of a concentration increment of 18 mg/L of $CaCO_3$. The lower range of phosphate measurements (0 to 0.5 mg/L) was divided into ten intervals, with an additional five intervals ranging up to twice that value. Iron (Fe) was tested with a kit (Dupla; Australia), dividing the range of concentration in six intervals (0, 0.005, 0.1, 0.2, 0.5, and 1 mg/L). An additional 1.5 L of water was collected and stored at 4°C for subsequent ex-situ analysis by the Seoul Research Institute of Health and Environment (Republic of Korea). Subsamples of this volume were analyzed for total nitrogen (T-N), total phosphorus (T-P), zinc (Zn), copper (Cu), mercury (Hg), cyanide (CN), cadmium (Cd), lead (Pb), ammonium nitrate (NH_3-N), nitrate (NO_3-N) and aluminum (Al). All

results were expressed as mg/L, and followed the standard methods of the APHA (Clesceri et al. 1996).

Statistical Analysis

We did not detect Hg, CN, Cd and Pb at any of the sites during any sampling period; thus, these four metals were excluded from statistical analyses. We coded the occurrence of each focal species as either present or absent at each site and tested for independence of occurrences of the two species using a Pearson Chi-square test. On this basis, we conducted separate analyses for each species with the water quality metrics measured at all sites.

We tested for the correlation between variables using Pearson correlations (n = 120): COH and PO_4 (r = –0.19, P = 0.04); PO_4 and Fe (r = 0.32, P = < 0.01); PO_4 and TP (r = 0.20, P = 0.03); TP and TN (r = 0.52, P < 0.01); NH_3 and TN (r = 0.31, P = 0.01); CU and Zn (r = 0.78, P < 0.01); Al and Zn (r = 0.21, P = 0.02).

Because all of the measured independent variables were continuous and the dependent variable for each species was dichotomous, we employed binary logistic regressions to determine which among the remaining water quality variables (temperature, pH, conductivity, COH, KH, Fe, PO4, TN, TP, Zn, Cu, NH3, NO3 and Al) influenced the occurrence of either *D. suweonensis* and *P. chosenicus*.

Table 11.1. Observed and expected counts for the occurrence of *Dryophytes suweonensis* and *Pelophylax chosenicus* at each of the 40 sites. The observed count is followed by the expected count in parentheses.

		Pelophylax chosenicus	
		Presence	Absence
Dryophytes suweonensis	Presence	14 (9.9)	4 (8.1)
	Absence	8 (12.1)	14 (9.9)

This was an appropriate analytical tool given that all observations were spatially independent for any one sampling date, and temporally discrete (i.e., observations made during one sampling period were assumed not to influence detections in subsequent sampling efforts). Another assumption for binary regression analysis is that the relationship between continuous variables and the logit transformation of the dependent variable should be linear (Box and Tidwell 1962). As such, we followed the Box-Tidwell procedure, and computed the logit transformation of all water quality-related variables. We then entered each variable in interaction with the corresponding logit transformed variable as covariate of the binomial regression. When assessing the significance of the linearity assumption, we applied Bonferroni corrections based on the number of terms entering the model (n = 14; Tabachnick and Fidell 2014). Based on these results (all $P > 0.004$), all continuous independent variables were linearly related to the logit of the dependent variable. All statistical analyses were conducted with SPSS v21.0 (SPSS, Inc., Chicago, USA).

RESULTS

Species Surveys

We detected *Dryophytes suweonensis* at 22 of our 40 sampling sites, whereas *Pelophylax chosenicus* was present at 18 of those site. If

one of the species was detected at a site, it was detected consistently at that site in all subsequent surveys. Both species occurred together at 14 sites. The occurrences of the two frog species were not independent of each other (Pearson Chi-square test; $\chi^2 = 6.86$; df = 1; $P = 0.01$). More sites were occupied by either frog species, or neither of them, than were occupied by only one of the focal species (Table 11.1; Fig. 11.1). Our survey efforts also detected non-target species: *D. japonicus* was present at all sites, *P. nigromaculatus* at 38 sites and *Bombina orientalis* at 3 sites.

Water Sampling

The binary logistic regressions used to discriminate the factors important to the occurrence of both *D. suweonensis* and *P. chosenicus* indicated three significant variables for each focal species (Table 11.2). Both iron and phosphate were correlated with the occurrence of the two species, although the values for the two variables were correlated (Pearson correlation; $r = 0.32$, $P < 0.01$, $n = 120$). Water conductivity was a significant predictor for the occurrence of *P. chosenicus*, whereas the amount of ammonium nitrate was a significant predictor for the occurrence of *D. suweonensis*. We did not detect either of the two focal species at sites having high values for water conductivity, and concentrations of phosphate and ammonium nitrate (Table 11.3).

Table 11.2. Results of logistic regressions for the occurrences of *Dryophytes suweonensis* and *Pelophylax chosenicus* at the study sites. Environmental contaminant variables were covariates in the models. Only iron (Fe) and phosphate (PO_4) are significant for both species, while conductivity was significant for *P. chosenicus* and ammonium nitrate (NH_3-N) was significant for *D. suweonensis*. Sample sizes are such that *D. suweonensis* was present at 22 sites and *P. chosenicus* at 18, out of a total of 40 sites. Numbers in bold font are significant at 0.05 level.

	Dryophytes suweonensis				*Pelophylax chosenicus*			
	mean ± SD	*df*	*P*	Exp(B)	mean ± SD	*df*	*P*	Exp(B)
Temp.	–0.05±0.15	1	0.74	0.95	0.25±0.15	1	0.08	1.29
pH	–1.07±0.89	1	0.23	0.34	–0.99±0.92	1	0.28	0.37
Cond.	0.09±0.5	1	0.86	1.09	–1.53±0.64	1	**0.02**	0.22
COH	0.4±0.57	1	0.48	1.49	–0.56±0.53	1	0.29	0.57
KH	–0.03±0.03	1	0.38	0.97	–0.01±0.03	1	0.84	0.99
Fe	3.54±1.5	1	**0.01**	34.54	2.95±1.31	1	**0.02**	19.17
PO_4	–0.68±0.34	1	**0.05**	0.51	–1.08±0.48	1	**0.02**	0.34
T-N	0.02±0.04	1	0.64	1.02	0.03±0.04	1	0.48	1.03
T-P	–0.06±0.63	1	0.92	0.94	–0.26±0.63	1	0.68	0.77
Zn	–38.92±30.49	1	0.20	0.01	1.27±17.32	1	0.94	3.55
Cu	–872.2.±30.6	1	0.99	0.01	–31.5±72.7	1	0.66	0.01
NH_3-N	–0.51±0.23	1	**0.03**	0.6	–0.06±0.14	1	0.65	0.94
NO_3-N	–0.66±0.67	1	0.32	0.52	0.75±0.74	1	0.31	2.11
Al	0.09±0.29	1	0.76	1.09	–0.47±0.38	1	0.22	0.62

Table 11.3. Descriptive statistics of the concentrations of contaminants of importance to frog occurrence in the study sites. The selection of contaminants is based on the results of logistic regressions, classified by sites with and without the focal species. Fe is divided in six classes ranging from 0 to 1 mg/L. PO_4 is divided in six classes ranging from 0 to 16 mg/L. NH_3 is in mg/L. Conductivity is in Siemens. Samples sizes are such that *Dryophytes suweonensis* was present at 22 sites and *Pelophylax chosenicus* at 18, out of a total of 40 sites. Data presented such as "mean ± SD".

	Absence	Presence
Dryophytes suweonensis		
Fe	0.10 ± 0.18	0.14 ± 0.24
PO_4	0.71 ± 1.68	0.26 ± 0.59
NH_3	1.42 ± 2.77	0.53 ± 0.98
Pelophylax chosenicus		
Conductivity	0.72 ± 0.52	0.50 ± 0.37
Fe	0.10 ± 0.16	0.15 ± 0.27
PO_4	0.68 ± 1.55	0.20 ± 0.54

DISCUSSION

Environmental contaminants have been cited as among the primary reasons for declines in amphibian populations (Alford and Richards 1999; Blaustein et al. 2003). Although we did not identify the source(s) of the water-borne chemicals detected at our study sites, the presence of phosphate and ammonium nitrate appears to be negatively correlated with the occurrence of *Dryophytes suweonensis* and *Pelophylax chosenicus*. In contrast, the presence of iron in water samples from these sites was positively associated with the occurrence of the two frog species. This latter finding was apparent in spite of the negative direct and indirect effects of metals documented for amphibians (Linder and Grillitsch 2000), and the acidification of the environment attributable to high concentrations of iron (Serrano et al. 2016).

Ammonium nitrate is toxic because of its role in converting oxygen carrying pigments into non-active forms; its toxicity is positively correlated with concentration and exposure time (Camargo et al. 2005). The relationship has been established in other frog species such as *Bufo gargarizans*, wherein tadpoles experience a delay in metamorphosis and higher death rate when exposed to high nitrate concentrations (Wang et al. 2015). One of the constituent elements, nitrogen, is naturally present in the environment, but currently in higher concentrations because of anthropogenic sources such as animal farming, urban and agricultural

runoff, industrial wastes, and sewage effluents (Bouchard et al. 1992; Vitousek et al. 1997; Carpenter et al. 1998). The fact that only *D. suweonensis* was affected in this manner might be related to a higher sensitivity, although no differences in this regard have been reported for the clades represented by the two focal species in our study (Egea-Serrano et al. 2012). Additionally, local variation in sensitivity to pollutants is known to occur in species within a clade (Pahkala et al. 2002).

Although the degree of toxicity can vary among species and life-history stages, phosphate is toxic to amphibians because of its interference with calcium metabolism (Odum and Zippel 2008). Phosphate can occur naturally through mineral weathering, but it originates primarily from fertilizers (Odum and Zippel 2008; Rohr et al. 2008; Vadas et al. 2015), such as glyphosate, which is broken down by water and sunlight into several components. Because of the strong impact of phosphate on amphibians, it seems reasonable to see fertilization of agricultural fields as one of the threats to which both *D. suweonensis* and *P. chosenicus* are subjected.

Pelophylax chosenicus was absent from sites where values for water conductivity were high. We suggest that this pattern is attributable to this more-aquatic species tending to remain submerged for long time periods (Ra et al. 2010). In contrast, *D. suweonensis* is described as being less aquatic in habit (Borzée et al. 2016c) and, as such, is likely to spend

less time in direct contact with the water. High conductivity levels are related to a decrease in fitness in some amphibians because of the influence on osmoregulation (Dodd 2010; Klaver et al. 2013). For example, high conductivity has been related to larval mortality in *Ambystoma maculatum* and *Lithobates sylvaticus* (Karraker et al. 2008).

Variation in the occurrence of either *D. suweonensis* or *P. chosenicus* was not attributable to pH and dissolved oxygen in our study, despite the stress that such conditions can impose on amphibian larvae (Dodd 2010; Zang et al. 2011). It is possible that other water contaminants are more closely related to changes in physiological processes and thus influence species occurrence. Previous studies have documented variable effects of pH on amphibian fitness (e.g., Beebee and Griffiths 2005; Dodd 2010). High concentrations of calcium and magnesium, related to conductivity, generally decrease the negative effect of metals and pH (Dodd 2010), besides being important for a range of physiological processes (Ultsch et al. 1999). The three non-target species of frogs observed at our study sites range widely in North-East Asia. Although additional research is needed to address these other taxa, variations in their occurrence could be explained by different tolerance levels for the metrics of water quality measured in our study (see Sparling et al. 2010).

The ranges of *D. suweonensis* and *P. chosenicus* are more restrictive, and the potential absence of these species from high-quality

habitats might reflect a dispersal limitation. The two species are already threatened by several factors, including landscape modification and emerging diseases (Kim et al. 2008; Borzée et al. 2017b), and they appear to be sensitive to some water pollutants as well. Of the water quality metrics that we measured, those that were most associated with species occurrence were also related to the agricultural practices. We suggest that, even though both *D. suweonensis* and *P. chosenicus* appear to breed successfully in wetlands modified for rice production, they are negatively impacted by isolated or sustained use of agricultural chemicals. A reduced use or ban of chemical use within the range of the two frog species would potentially prevent their population decline.

Chapter twelve

TRANSLOCATION OF THE ENDANGERED AND ENDEMIC KOREAN TREEFROG *DRYOPHYTES SUWEONENSIS*.

ABSTRACT

Endangered species in heavily modified landscapes are most likely to go extinct if no conservation plan is implemented. The Suweon Treefrog (*Dryophytes suweonensis*) is an endemic and endangered species from the Korean Peninsula. In an attempt to preserve the species a translocation plan was implemented in the city of Suwon. The site was a specially terraformed island in a reservoir. Egg clutches were collected from four nearby sites, and were hatched and head-started in laboratory in 2015. A total of 150 froglets were released at the site, and the remaining froglets were released at their original collection site for population strengthening. The translocated population was monitored in 2016, when calling male *D. suweonensis*, tadpoles and juveniles were identified. Juveniles were seen until the last week before putative hibernation. However, a single male was recorded calling in 2017. The population was consequently considered functionally extinct. Failure of the translocation may arise from mismanagement of the vegetation surrounding the wetlands. The project benefited the original capture populations through reinforcement, allowed for the development of rearing protocols for the

species, defined ecological requirements for the species, but cannot be qualified a translocation success.

INTRODUCTION

Due to the threatened status of a high number of amphibian species, in-situ and ex-situ conservation programs are being established around the world (Stuart et al. 2004, Pimm et al. 2014). Each type of conservation plan has advantages and drawbacks, but when possible, the translocation of a few individuals in a restored habitat can make a difference towards the survival of the species (Zippel et al. 2011). However, the success rate is usually low, partly because of the lack of report on non-successful methods to the scientific community (Griffith et al. 1989; Dodd Jr and Seigel 1991; Germano and Bishop 2009).

The Suweon Treefrog (*Dryophytes suweonensis*) is an endangered species endemic to the Korean peninsula (IUCN 2017a), although potentially synonymous to *Dryophytes* (=*Hyla*) *immaculatus* from China (Dufresnes et al. 2016; Borzée et al. 2017a). *Dryophytes suweonensis* is an important evolutionary species as displaying a rare ZW karyotype that is not shared with its sister species *D. japonicus* (Dufresnes et al. 2015). It is therefore important to develop conservation plans to prevent the extinction of the species. Here, we attempted reintroducing *D. suweonensis* at a site in the city of Suwon. The site was terraformed

following the ecological requirements of the species, to avoid the shortcomings that lead to the extirpation of the species in its eponymous city, where it was described in 1980 (Kuramoto 1980).

MATERIALS AND METHODS

Translocation site

This work was conducted under the permit 2015-4 issued by the Ministry of Environment of the Republic of Korea. The translocation site was an island covering 6 600 m^2, located at the northwest corner of Ilwol Reservoir in the City of Suwon in the Republic of Korea (37.289863° N; 126.970954° E; Fig. 12.1). This site is owned by the Korea Rural Community Corporation (KRCC) and was chosen for this project as the result of the allocation of a restoration grant to KRCC by the Ministry of Environment. Unfortunately, the location of the translocation site was determined without feasibility analyses and risk assessment (IUCN/SSC 2013), prior to the project. The north and west corners of the site were isolated from the surrounding paths by water collection channels, and the south and east corners of the site were facing the waters of the reservoir. Besides, the site is adjacent to 8-lane roads on its northern and western side, and to urban areas to the south and east, making it isolated from the range of the species. The site was designed to feature all environmental variables known to be required by *Dryophytes suweonensis* at that point in

time.

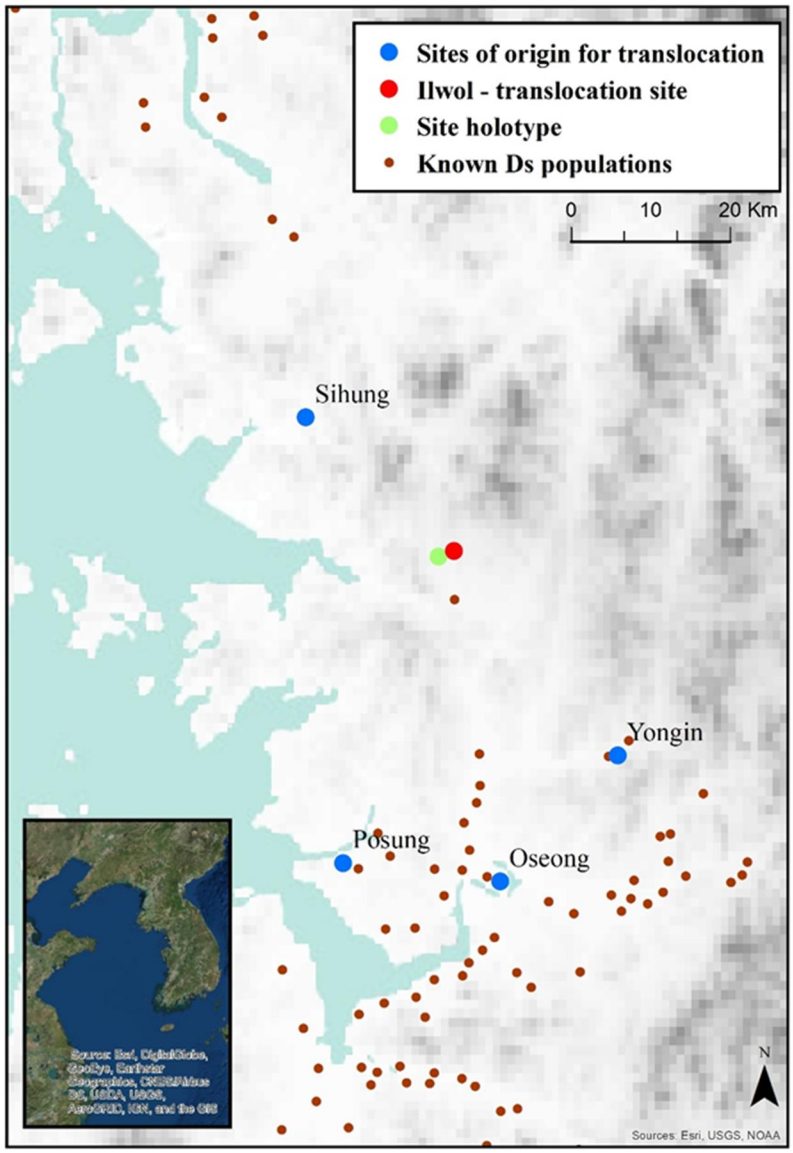


Figure 12.1. Geographic illustration of the sites at which egg clutches were collected, the site where individuals were released in Ilwol reservoir in Suwon, and the site where *Dryophytes suweonensis* (= Ds) was described. Map was generated with ArcMap 9.3 (Environmental Systems Resource Institute, Redlands, California, USA; http://www.esri.com/).

The island was transformed to encompass a rice paddy covering 580 m^2 and two ponds (Fig. 12.2) with vegetation cover (lotus; *Nelumbo nucifera*), and a shallow pond with reed banks (*Typha* spp.).

Tall trees (> 5 m) were planted to supplement the tree cover already present at the site. The plant species selection was based on the only known association between the *D. suweonensis* and a tree species: the Korean willow (*Salix koreensis*; Borzée and Jang 2015). Finally, as the hibernation ecology of the species was not known at the time, and because breeding sites of *D. suweonensis* are almost always surrounded by forested hills, a low hill planted with pine trees (*Pinus* sp.) was set on the northern edge of the island. Cut wood and stone stacks were also set in the vicinity of the rice paddy to help with sheltering during brumation and hibernation. The reed bed at the south of the island was there originally and was not modified as not assessed to be negative to the translocation of *D. suweonensis*.

Collection sites selection

To found a population, Albert et al. (2015) recommend mixing animals from multiples sites, while avoiding the genetically most divergent populations. Mixing lineages also showed rapid population growth and increase in heterozygosity in several amphibian species (Madsen et al. 2004; Vilà et al. 2003; Johnson et al. 2010; Spielman et al. 2004a). The collection sites selected for sampling were thus in Sihung,

Yongin, Posung and Oseong (Fig. 12.1). The choice of collection sites for translocation was based on species' range-wide surveys (Borzée et al. 2016c; Borzée et al. 2017b) and genetic analyses (Borzée et al. 2015b). The sites selected as the origins of translocated individuals were within the four closest sites to the translocation site, and displayed the highest genetic diversity when combined, despite low variations between isolated populations. The analysis was based on the sequences of 578 bp of CO1, based on the protocol developed by Jang et al. (2011). The collection sites selected included 63.41% of the totality of CO1 haplotypes recorded for the species, while including 89.65% of haplotypes from the South-Seoul metapopulations, to which the site belongs. Thus, sampling an egg masses issued from two unrelated parents from any population had the potential to cover 34.48% of the haplotypes from South-Seoul metapopulations. This was assessed to be adequate as the highest genetic diversity at a site was 24.1% of haplotypes. This selection enabled maintaining the genetic structure of the species, while increasing the genetic diversity through cross-population breeding in the F1 generation.

Field surveys at collection sites: Surveys were conducted at the collection sites in 2015. The survey schedule was set as 3 March 2015, 4 and 21 April 2015, five surveys in May 2015 with about 5-day interval between successive surveys, weekly surveys in June 2015, and surveys on 3 and 7 July 2015. This schedule intended estimating temporal phenology

and population size at all sites.

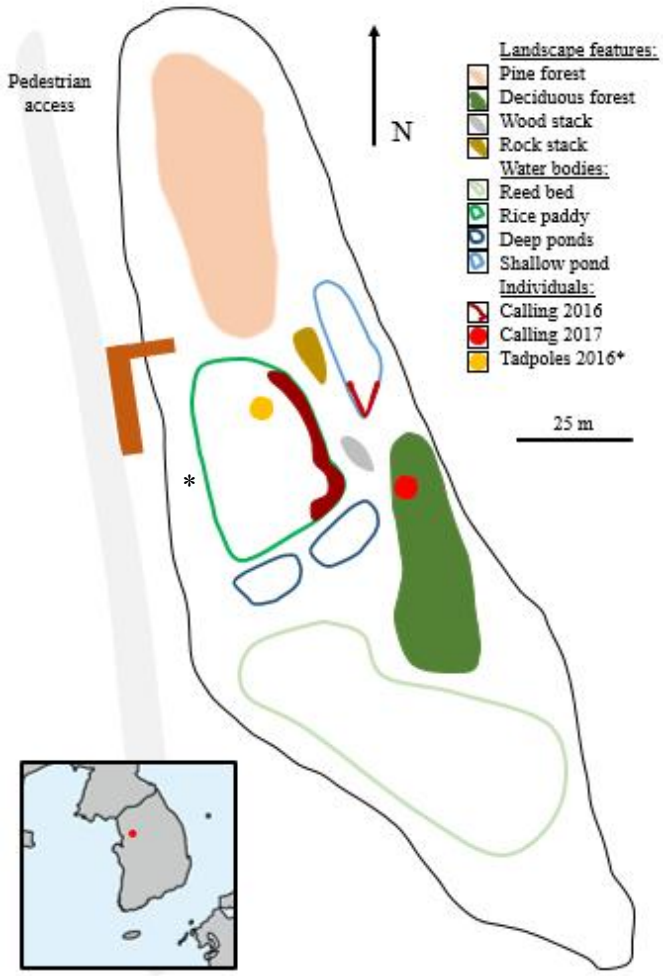


Figure 12.2

Figure 2. Terraformed island where 150 froglet *Dryophytes suweonensis* were released. Release took place on the eastern edge of the rice paddy in three separate events between early July and late August 2015. The red dot on the inset map describes the spatial location of the site within the Republic of Korea (37.289623°N, 126.971062°E). *denotes where the tadpole was caught in 2016, although individuals were seen in the totality of the rice paddy.

The translocation site in Suwon, and the closest site to the one where the species was described (Kuramoto 1980), were also surveyed at these dates to ensure ecological variations matching with the ones at sites where the species was known to occur, and to assess future potential reintroductions (Fig. 12.1). Monitoring was conducted, following the protocol described in Borzée et al. (2017b) and Borzée et al. (2017c). At each site, we collected abiotic variables including luminosity (Lx), air temperature (°C), relative humidity (%) air pressure (hPa), water temperature (°C), water conductivity (μS), water pH, the number of calling individuals for *D. suweonensis* and the Calling Index (CI) for *D. japonicus, Pelophylax nigromaculatus*, *Kaloula borealis*, *Bufo gargarizans,* and *Rana coreana*. Here, the CI was the number of calling individuals is classified in four categories: 0: no individuals are calling; 1: calls are not overlapping and it is possible to count the number of individuals calling; 2: calls are overlapping and it is still possible to count the number of individuals calling; 3: it becomes impossible to count the number of individuals (Mossman et al. 1998; Weir et al. 2005; Roh et al. 2014).

Eggs collection: Sampling of egg clutches for translocation was conducted after sunset in spring 2015. We visually searched for amplexed pairs at the four collection sites. The localization of amplexed individuals was facilitated by advertisement calls. Once found, the amplexed pairs

were cautiously caught and transferred into an aerated plastic boxes (60x40x40 cm– WxLxH) and left in the rice paddy where they were found until morning. The amplexed pairs were released at the point of capture on the next morning, immediately after the collection of oral and skin swabs, following the protocol from Borzée et al. (2017c). A single difference in the protocol was made: the two individuals from the pair were considered to be two replicates of a single unit during the subsequent test for diseases

Once brought back to the laboratory, eggs were set into 30x30x30 cm glass tanks in the water from the rice paddy where they had been caught. Water change was avoided until hatching to facilitate the highest rate of hatching. Each egg mass was accommodated separately, with a standard air stone for aquarium set at lowest bubbling rate to aerate the water but prevent the eggs from drifting. Once hatched, the tadpoles were kept together until *circa* stage 26 (Gosner 1960), when they were divided by groups of 50 individuals, and transferred to new 30x30x30 cm glass tanks.

Tadpole rearing: The newly transferred tadpoles were accommodated with 30% of water originating from their rice paddy and 70% of carbon filtered and subsequently 72-h aged tap water. A third of the water volume was then changed weekly from the bottom to remove decaying matters. The water used for changes was also filtered and 72 h aged water, until metamorphosis of all tadpoles. In the tanks, the average conductivity was

230 ± 167 µS (mean ± SD), the pH was 8.3 ± 0.9; and the temperature was 16.4 ± 4.2 °C the first week but 26.3 ± 2.4 °C during the last week as all aquariums were kept at environmental temperature by keeping all doors and windows open. Air stones were provided to each tank for oxygenation and to prevent bacterial multiplication.

Tadpoles were fed *ad libitum* on a boiled and blended mix of Chinese mallow (*Malva verticillata*) and animal nutrients from NovoTab fish food pellets (43% crude protein; JBL, Neuhofen, Germany). Each tank was illuminated by an LED strip, set on the same cycle as the natural photoperiod, and adjusted bi-monthly to follow natural variations. Once an individual had developed both front legs, it was transferred to another set-up to finish metamorphosis without risk of predation by siblings. Tanks were checked twice daily during the period of metamorphosis.

Froglets rearing: Metamorphosing tadpoles were transferred to terrariums (Exo Terra Glass Terrarium 30x30x30; Colorado, USA) with lateral opening, and mesh ceiling for aeration. Each terrarium was set with a layer of wet towel changed twice a week, a petri dish filled with water, and sprayed daily to maintain > 70% relative humidity, but no flooded area. Each terrarium was illuminated with a UV-B bulb (PT 2186; Exo Terra Repti-Glo 5.0; Colorado, USA), temporally matching with natural sunlight, and readjusted every second week. Doors and windows of the rearing room were kept open to maintain natural atmospheric variations.

The first week was on average 24.3 ± 1.7 °C, with the maximum reaching 36.7 °C later in the season. A wooden rod (diameter = 5 cm) was placed diagonally from the left front bottom to the back right top in each tank for froglets to climb on.

Individuals transferred from the aquariums were first deposited into petri-dishes, where they usually stayed until tail retention. The water in petri dishes was changed every other day, or daily when needed. Terraria were not populated with more than 20 individuals, to keep low density, and prevent important size variation between individuals. This was done to avoid exploitative competition for food items. The youngest feeding individuals were fed daily *ad libitum*, starting with the equivalent of five pinheads or fruit flies per individual. As soon as size allowed, individuals were fed with crickets of larger size, complemented with small worms. All food items were first powered with calcium and vitamins (Powder Supplement PT1850 and PT1860; Exo Terra, Colorado, USA).

Pre-release screening: Prior to release, all individuals were tested for a set of diseases, including Chytrid fungus (*Bd*) and ranaviruses. Diseases detection was conducted through molecular analyses of skin swabs, for every tenth individual randomly picked, and all dead individuals. Swabbing was done with sterile fine-tip swabs (Medical Wire & Equipment Co Ltd; Corsham, UK), wearing a new pair of vinyl gloves for each frog. Frogs were systematically swabbed five times on each toe of

the hind legs, each foot, the inner thighs, and both sides of the abdomen. Swabs were stored in individual 1.5 mL tubes at – 20 °C, until analysis.

Bd detection was conducted together with the samples presented in Borzée et al. 2017c, and thus follows the same protocols, primers, and controls. Ranaviruses were tested following the protocols set by Mao et al. (1997) and the PCR run times described by Greer and Collins (2007). The PCR amplification relies on primers specific for a 500 bp fragment of the Ranavirus major Capsid Protein. Ranavirus had not been detected in Korea at the time of testing, but has been detected since then in *D. japonicus*, *K. borealis* and *R. huanrenensis* (Park et al. 2017; Kwon et al. 2017), and is thus likely to be carried by *D. suweonensis*.

Release: Individuals were released after 8 pm to ensure decreased predation by potential birds and reptiles present at the site. In three events, individuals were housed in plastic containers by groups of five to ten. The containers were distributed at regular interval on the eastern edge of the rice paddy (Fig. 12.2), where the vegetation was the most abundant. Releases took place in three waves in order the match the rhythm of metamorphosis of tadpoles in the lab, and keep metamorphosed individuals for the shortest period possible. All released individuals were old enough to feed on live preys. The first release took place on 15 July 2015, with 20 individuals released. The second release (50 individuals) was on 4 august 2015 and the last release (80 individuals) was on 25

August 2015.

Monitoring: Subsequently to the release in 2015, the site was surveyed every 3 days until the average temperature dropped below 20 °C. From this point, surveys were conducted weekly, until 21 October 2015, when the rice paddy was harvested. The site was also surveys in 2016 and 2017, with surveys conducted monthly in February and March, twice in April, weekly from the first week of May to the last second week of August, and then bi-weekly until the first freeze in the second week of November in 2016, and until the last week of September in 2017. During surveys, the temperature (°C), relative humidity (%), air pressure (hPa), water temperature (°C), water conductivity (μS) and pH were collected, as well as calling index, or individual count if possible, for all anuran species present at the site.

RESULTS

Survey at collection sites: The amphibian community of the translocation site comprised seven species. *Dryophytes japonicus* and *Pelophylax nigromaculatus* were present at all sites, but *Kaloula borealis*, *Bufo gargarizans* and *Rana coreana* were detected in Ilwol only. The other species detected were *D. suweonensis* and *P. chosenicus*, at all four collection sites.

Dryophytes suweonensis started calling in Posung (Fig. 12.1) the second last week of April, and was then heard calling at the four collection sites from the second week of May. The highest number of calling individuals was recorded in Oseong, where the calling activity was also the longest, with until 3 July 2015 (Fig. 12.3). We also assessed the diel calling activity of *D. suweonensis*. The peak calling activity was observed between 19:00 and 19:30, with the earliest calls detected at 16:00 and the latest at 1:30 am on the next day (Fig. 12.4). The highest number of calling individuals at a single site was 13 individuals, recorded in Oseong, while the average number of calling individuals was 3.8 (± 1.2; Fig. 12.4).

Finally, we measured each abiotic factor for each level of advertisement call production, here defined as the total number of individuals calling at a site (0 to 5), or if > 10 individuals. We did not record between six and nine individuals calling at any of the sites (Table 12.1). The highest number of calling individuals was detected below 20 ˚C, although up to four individuals at a time were surveyed at the highest temperature (> 24 ˚C; Fig. 12.5). The air humidity displayed only minor variations through the range of the number of calling individuals, with the lowest points matching with the lowest and highest number of calling individuals (Fig. 12.5). The air pressure displayed the opposite pattern to the air humidity, with the highest values matching with the lowest and highest number of individuals calling (Fig. 12.5). Regarding the abiotic

variables related to water quality, the water temperature was at the highest point when four individuals were calling. The pH and conductivity did not have any visible effect on the number of calling individuals. Finally, low rain and luminosity were correlated with the highest number of calling individuals (Fig. 12.5).

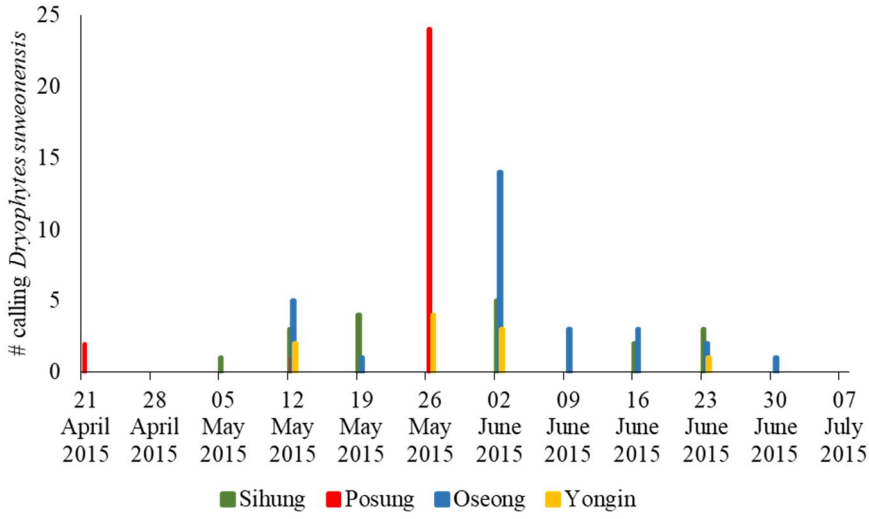


Figure 12.3. Seasonal calling phenology for *Dryophytes suweonensis* at the four sites where eggs were collected for translocation to the site in Suwon. The species was not detected in Ilwol neither than the site where it was first described. The surveys between 03 March and 14 April are omitted from the graph as no individual was found calling.

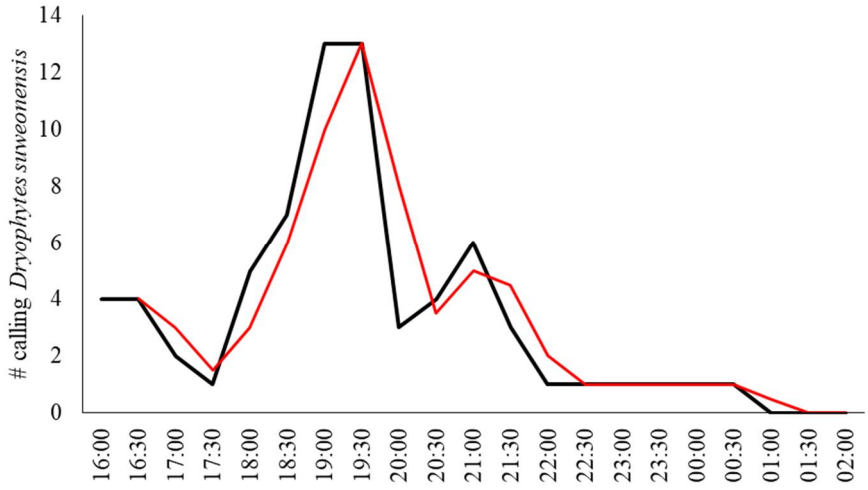


Figure 12.4. Diel calling activity for *Dryophytes suweonensis*. The data is summarized from all sites and all surveys. The black line is the number of individuals and the red line the averaged number between two time points. The peak in the number of calling individual is right after sunset. 16:00 is post-meridiem and 02:00 is ante-meridiem of the next day.

Tadpoles and froglets rearing: During the rearing period, water quality tests did not detect excess of nitrates nor ammonium. There were at least five replicate tanks for each family, and up to eight tanks for the largest egg clutch. Tanks were inspected daily for dead individuals, which seldom happened. A few cases of necrophagy were detected, and in this case the dead individual was not removed and totally consumed by siblings. This decision was taken to enable tadpoles to access any resource that would not have been provided by the diet. It is unclear if these cases resulted from cannibalism or if the individuals were dead before being preyed on. Cannibalism was however observed, with several individuals preying on a sibling, typically feeding first on the tail, and then on guts once the individual preyed upon became immobilized.

Metamorphosing individuals transferred to the petri dish were usually clumsy, and several cases of death because of drying, or drowning, were reported. Some individuals reaching dry areas were sometimes not able to get back to the humid zones and died, and some individuals did not seem to see the transparent edge of the plastic petri dishes and swam straight against the plastic without moving upwards, until death. This was corrected by sticking opaque tape on the edge of the petri dishes once the problem understood. An estimated upward to 60 metamorphosing individuals died as the result of the problems mentioned above. A few cases of abnormal development were detected, such as missing limbs ($n <$

5 out of > 1 200 individuals) and missing eyes (n < 10 out of > 1 200 individuals). All dead individuals were preserved in 100% alcohol and frozen for further testing.

The survival of froglets was *circa* 75%, with *circa* 250 dead individuals retrieved in total. Death was associated with bloating, lack of activity, lack of appetite, and increasing weakness. Baths in Ringer's solution, an isotonic in comparison to the body fluids of amphibians, did not seem to improve survival but seemed to delay death. All healthy individuals were active and preferentially found on the wooden support in the terrariums. All dead individuals were also preserved in 100% alcohol and frozen for further testing of diseases.

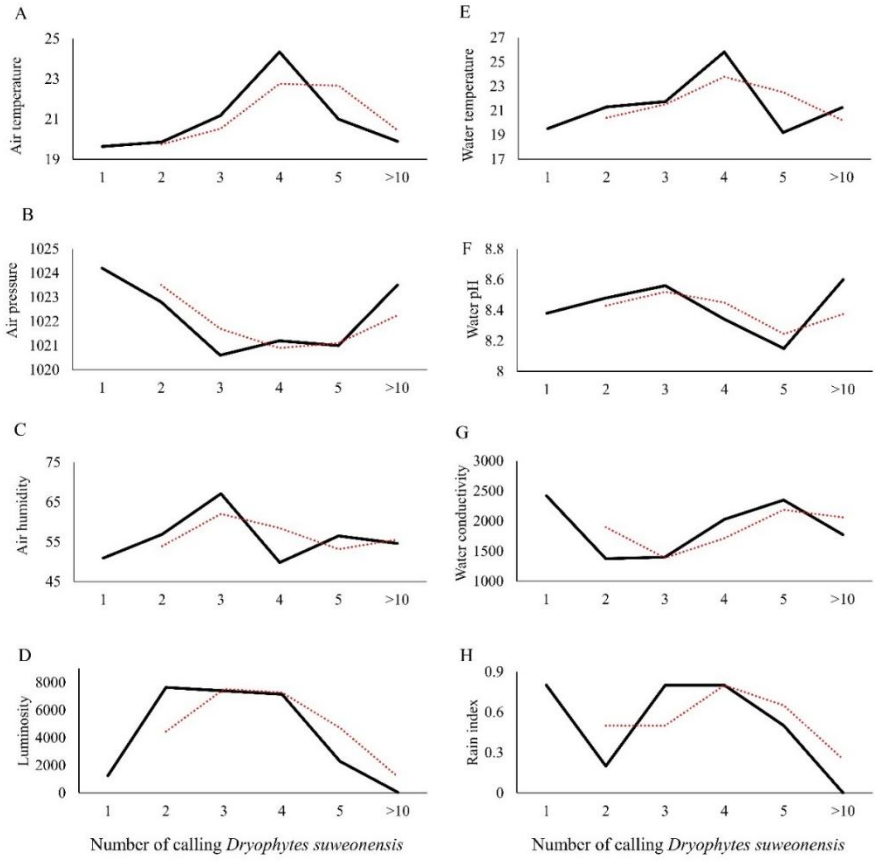
Pre-release screening: Before release, all individuals were visually inspected for lesion and apparent handicap. Testing for *Bd* and ranavirus were negative for all replicates, for both randomly picked and dead individuals, swabbed post-mortem. A few weak looking individuals were kept from being released and were instead released with the next wave, once adequately active.

Release and monitoring: A total of 150 individuals were released at the translocation site, and all remaining froglets were released at their site of origin for population strengthening. At the translocation site, individuals were detected on a regular basis for a year.

Table 12.1. Average values for abiotic variables in relation with the number of calling *Dryophytes suweonensis*. We did no record between 6 and 9 individuals calling at any of the sites.

# *D. suweonensis*	1	2	3	4	5	>10
Air temperature (°C)	19.64	19.86	21.18	24.34	21	19.9
Air humidity (%)	50.9	56.9	67.12	49.82	56.5	54.6
Water temperature (°C)	19.52	21.3	21.74	25.82	19.2	21.25
Water acidity (pH)	8.38	8.48	8.56	8.34	8.15	8.6
Rain index (1 to 4)	0.8	0.2	0.8	0.8	0.5	0
Luminosity (lx)	1252.6	7637.6	7393.5	7149.4	2292	41
Pressure (hPa)	1024.2	1022.8	1020.6	1021.2	1021	1023.5
Water conductivity (µS)	2419.6	1375	1400.8	2026	2347.5	1774.5

Figure 12.5. Variation in air and water related abiotic variables in relation to the calling activity of *Dryophytes suweonensis* at the four capture sites. Relation between the number of calling *D. suweonensis* and air temperature (A), air pressure (B), air humidity (C), rain index (D), water temperature (E), water pH (F), conductivity (G) and luminosity (H). The black line is the number of individuals and the red line the averaged number.



Out of the 23 surveys conducted post-release in 2015, *D. suweonensis* was reported in varying numbers for 13 surveys. The maximum number of individuals observed in a single survey was 17. The main factor affecting the number of individuals observed was the temperature, with no individuals seen when the temperature dropped below 17.6 °C, and the maximum number of individuals was found between 23 and 30 °C (Fig. 12.6).

Weekly surveys in 2016 detected seven calling males on 19 May. Males were heard calling for seven weeks, and the peak calling activity involved 11 calling males on 21 May. Tadpoles were seen in the rice paddy from the second week of June, and one individual, remarkable because of its red tail, was caught and tail-clipped for molecular identification through CO1 sequencing (protocols from Jang et al. 2011). The sequence acquired had a 98.2% match with *Dryophytes suweonensis* from GeneBank, and thus demonstrates breeding by *D. suweonensis* post translocation. Newly emerged froglets were encountered from 17 July 2016.

There were several managerial mistakes in the translocation site in 2016. Grass was mowed subsequently to the emergence of froglets in June 2016, around the rice paddy, and where all froglets and metamorphs had been surveyed.

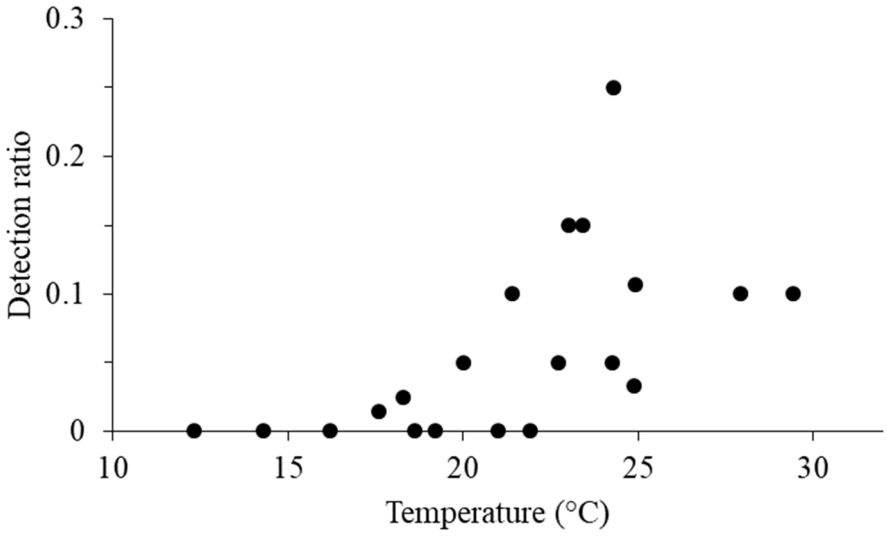


Figure 12.6. Detection of froglet *Dryophytes suweonensis* in relation with temperature. The main factor affecting the number of individuals observed was the temperature, with no individuals seen when the temperature dropped below 17.6 °C, and the maximum number of individuals between 23 and 30 °C.

It also resulted on the "clean-up" of the site, with the hibernation sites (Fig. 12.2) removed, the rice straws burnt on top of the "natural compost pile", which also happened to be the place where individuals were last sighted in autumn 2016. Some dead trees were also replaced for "maintenance" of the site. A single calling male was detected on 25 May 2017, and not in the rice paddy but in the flooded forest south of the rice paddy (Fig. 12.2), following heavy rains and abnormally high water levels at the reservoir where the island had been built.

DISCUSSION

Understanding the ecology and evolution of an endangered species is vital for its effective protection and conservation (Young 2000; Spielman et al. 2004b). Here, we demonstrated that translocation of *Dryophytes suweonensis* froglets has the potential to succeed, and that both sexes are sexually mature, with males producing advertisement calls, within a year. However, research and management teams need adequate and constant communication, with joint effort and participation to ensure the success of this type of project. Nevertheless, we argue that this project had a positive impact, other than the translocation, as knowledge on the ecology of the species was acquired, and population strengthening at the four sampling populations of origin was completed unhindered.

Conservation programs for amphibians are numerous, *in situ* as

well as *ex situ* (Zippel et al. 2011), mostly driven by exacerbated population decline over the last decades (Stuart et al. 2004). However, the setting of a conservation program entails the *a priori* knowledge of the genetic landscape of the focal species in order to preserve genetic diversity and allow for genetic exchange (Yan et al. 2013). In this regard, the selection of the translocation site was considered an adequate choice because it establishes a stepping-stone population between the disconnected populations south and north of Seoul (Borzée et al. 2015b). Besides, the site was within the range of ecological preferences of the species.

For further project of this type, we recommend the removal of any potential predator, even unlikely, as a ground skink (*Takydromus* spp.) was seen catching and eating one of the released individuals within 10 seconds of jumping out of the release box. We expect other predation events on the froglets released as some Tiger keelbacks (*Rhabdophis tigrinus*) were found at the site. Some unexpected additional benefits of this projects were the breeding activity of endangered *Rana coreana* and *Kaloula borealis* at the site, as well as the common Asian toad (*Bufo gargarizans*) and the fire-bellied toad (*Bombina orientalis*).

Chapter thirteen

POPULATION VIABILITY ANALYSIS FOR THE ENDANGERED *DRYOPHYTES SUWEONENSIS*.

ABSTRACT

Numerous species are under threat of extinction, and the proportion of such species is comparatively higher in amphibians. Assessing the point at which a species will become functionally extinct is hard to pinpoint, although population viability tools provide extinction probability with an accuracy correlated to the one of the data fed into the models. This is for instance the case of the VORTEX software. We used this software to run a Population Viability Analysis for the Suweon Treefrog (*Dryophytes suweonensis*), an endangered and endemic species to the Korean Peninsula. All the data available for the species were integrated in the model. The results, based on an estimated original population size of 2525 individuals, showed that the population will drop below 1000 individuals within nine years, below 500 within 20 years, and the species' probability of extinction within 100 years was 1.00. The median time of first extinction (n = 1000) for *D. suweonensis* was 9.98 years. We therefore call for the development of the utmost conservation efforts to prevent the extinction of this evolutionary significant species, homonymous to the Korean city where it was described.

INTRODUCTION

The extinction of species, especially amphibians, is a presently recurrent subject. Most extinctions are not directly witnessable, or are acknowledged only years after the loss of the species (Reed 1996; Nichols et al. 1998; MacKenzie et al. 2003). An exception is the Rabbs' fringe-limbed tree frog (*Ecnomiohyla rabborum*; Mendelson et al. 2008). To the best of our knowledge, the species went extinct in the wild due to the Chytrid fungus (Mendelson 2011), and the last captive individuals are dying in captivity at Atlanta Botanical Garden, making current generations direct witnesses of the loss of this species.

Population Viability Analyses (PVA), as defined by Gilpin (1986), are predictive analyses of the survival of a population over a given period of time, in the probabilistic sense (Shaffer 1990). PVAs require the evaluation of data and models and predict the probability of persistence for a given population at a given future time point (Boyce 1992). As a result, it is a type of risk analysis for the survival of a population (Ginzburg et al. 1982). However, the results of PVAs differ based on the ecology of the species, the data available for the population(s) and the modeller's abilities (Boyce 1992). Besides, the PVA tools provide an extinction probability only as accurate as the data fed into the models (Staples et al. 2004).

The Suweon Treefrog (*Dryophytes suweonensis*) is an endangered species (IUCN 2017a) endemic to the Korean Peninsula (Borzée et al. 2017b). The species on the Korean Peninsula is divided into 14 disconnected populations (Borzée *Chapter 2*), with a total population size between two and three thousand individuals (Borzée and Jang *in prep* - *Chapter 3*). The species is currently found breeding solely in rice paddies (Borzée and Jang 2015), because of habitat destruction, and is submitted to other threats such as competition (Borzée et al. 2016a; Borzée et al. 2016b), water contamination (Borzée et al. 2017d) and invasive diseases (Borzée et al. 2017c). Based on the literature available, the species is already doomed in the long term, as the population size is likely below the minimum effective population size for evolutionary potential (Frankham et al. 2014). This is however hopefully not accurate, as seen through the case of cheetah (*Acinonyx jubatus;* Castro-Prieto et al. 2011) or southern elephant seals (*Mirounga leonine;* Slade 1992), which survive despite very low genetic diversity. Besides, conservation actions can help provide evolutionary potential, such as for instance through translocations (Wright et al. 2014). The objective of this research project was to assess the viability of *D. suweonensis*, through the use of the software VORTEX (Lacy and Pollak 2017).

MATERIAL AND METHODS

All the data used in this study was extracted from the literature, or from yet non-published data, as specified. No specific timeline is required for PVAs (Boyce 1992), and we arbitrarily selected population size predictions at several key points, such as 1000 individuals, and key times, such as 100 years from now.

This analysis was conducted on the software VORTEX 10.2.5.0 (2017 IUCN SSC Conservation Breeding Group & Chicago Zoological Society; Chicago, USA), running stochastic simulations of the extinction process. Data input was conducted such as detailed by the user's manual guide, except when specified (Lacy et al. 2017).

All the sites where the species is known to occur in the Republic of Korea (Borzée et al. 2017b) where surveyed in 2015, 2016 and 2017 to estimate the population size at each of the site. Sites were pooled into clusters when within 200 m and not separated by barriers (i.e. seasonal migration potential; Borzée *Chapter 8*; Roh et al. 2014). If clusters where within dispersion distance, here 10 km (Smith and Green 2005; Angelone and Holderegger 2009; Roh et al. 2014), they were then pooled into population. Thus, 14 independent populations were detected (Borzée *Chapter 2*). The total number of calling individuals was assessed for each population (Borzée and Jang - *Chapter 3*), to which, a number of females was added independently for each population, based on data for *D.*

japonicus collected in 2014 (Kim 2015a). In that study, the least biased sex ratio was 2.53:1 (males:female) while the most biased was 19.00:1. During the lekking period, the average sex ratio was 9.05:1, signifying that 90.05 % of individuals at the breeding site were males. Thus, we added 9.95 % of the number of individuals to the number of individuals detected at each of the sites. This value was used as initial population size (Table 13.1). We acknowledge the match with other study, as well as the large variation, such as compared to Friedl and Klump (1997), where the sex ratios were 1.52:1 and 2.15:1. Dispersion between populations was set at 0 %, as the definition of populations here reflects the isolation of the 14 populations. The percentage of males in the breeding pool was set at 90.05, because all males were breeding males as our surveys were based on the detection of calling individuals. Individuals were equally distributed in each age class for each of the sites, based on the initial population sizes.

The species was set as polygynous, with the age at first offspring for males and females set at 1 year (Borzée et al. *in review-b*). We use the data provided by Friedl and Klump (1997) for the maximum breeding age, 6 years, as the two species are ecologically similar and no data is so far available for *D. suweonensis*. The maximum number of broods per year was one, as a single female out of 213 was found at the breeding site more than once for *D. japonicus* by Kim (2015a). The

average number of progeny by brood was 358, extracted from Borzée et al. (*in review-b*), where the number of egg per clutch was 344 , 412, 278 and 398, and the sex ratio at birth was set as 50:50, due to the lack of adequate knowledge. The probability of breeding at low density was 0.5, and the probability of breeding at carrying capacity was disregarded here as the carrying capacity of sites is not reached at any of the clusters. The probability of success in breeding was 90 % per female, with 10 % assigned to 0 brood. The number of offspring per female per brood was in average 358 (SD = 10 %), and following a normal distribution. Standard deviation was arbitrarily set at 10 % as there is very little variation possible in rice paddies, the standardised habitat the frogs currently have to use for breeding.

The mortality rate was set at 95 % between 0 and 1 years (SD = 10 %), as tadpoles are numerous but only few manage to survive predation, then through metamorphosis, and then the first hibernation (Wilbur and Collins 1973; Hine et al. 1981; Altwegg and Reyer 2003; Gonzalez et al. 2011). The mortality after age 1 was 60 % (SD = 5) for females, and 70 % (SD = 10) for males. Mortality was set this way as the species brumates and hibernates in rice paddies, which are harvested and burnt (Borzée *Chapter 8*). It was set even higher for males as they stay in the vicinity of rice paddies throughout the breeding season and are exposed to the mowing of weed when resting on banks (Borzée et al.

2016a) and exposition to chemicals (Borzée et al. 2017d).

Table 13.1. Input data for the VORTEX model. The name of the populations is provided to simplify the development of conservation effort. All values are rounded to the upper full unit.

	Population	Surveyed population size (male)	Number of females added
Buyeo	1	49	5
Chungju	3	79	9
Wonju	4	69	8
Icheon	5	61	7
Jincheon	6	8	1
Asan	7	1372	152
Suweon	8	1	1
Gimpo	9	4	1
Haseong	10	8	1
Gangwha	11	74	8
Paju	12	176	19
Nonsan	13	10	1
Iksan	14	359	40
Sihung	15	6	1

Three types of catastrophes were set: encroachment, drought and agricultural conversion of rice paddies to dry agriculture. Encroachment was calculated to occur at a frequency of 22.33 % of sites, data extracted from Borzée and Jang (*in prep - Chapter 3*). The frequency for drought and paddy conversion was set at 14 %, based on the reference provided by the software (Reed et al. 2003), a high frequency, related to increasing droughts because of climate change, and the decreasing value of rice in comparison to other crops.

Besides, 30 % of the population was set as harvested every year, because (1) numerous birds (e.g. *Nycticorax nycticorax*, *Egretta alba*, *E. cinerea*, *E. garzetta*, *Mesophoyx intermedia*…) and snakes (*Rhabdophis tigrinus*) are seen preying on adults; and because (2) 10.52 % of individuals with mtDNA assignment to *D. suweonensis* were found to be in cytonuclear disequilibrium and are thus removed from the species' genepool (Borzée *Chapter 9*). No supplementation was selected, and no data was entered for genetics

The inbreeding depression was left such as suggested, with lethal equivalents set at 6.29, based on O'Grady et al. (2006), despite the known effect of inbreeding on the fitness of the *D. suweonensis* after the first generation (Borzée *Chapter 4*), but the inability to calculate it in the way that could be integrated into the model. The environmental variation correlation among populations was set at 0.9 as there is very little

variation between populations. It was however not set as 1 as sites at the southern localities are harvested twice a year, once for rice and once for barley, and the effect of the agricultural practices on the populations is not known yet.

The model was set at 1000 iterations for 100 years, with 365 days per year. Data points for probability of extinction and population size were extracted for the metapopulation from the output data every 5 years to be subsequently plotted for simplified visualisation of results.

RESULTS

The results of the PVA for *Dryophytes suweonensis* ran in vortex with 1000 replicates for 100 years and an estimated original population size of 2525 individuals gave a probability of extinction of 1.00 (SD = 0.00). The probability of survival went below 10 % after 55 years (Fig. 13.1). The median time of first extinction for the species was 9.98 (SD = 5.81) years, while the average was 30.25 years (SD = 18.51; Fig. 13.2).

Besides, the results showed that the population will drop below 1000 individuals within nine years and below 500 within 20 years, the threshold under which species are typically suspected to be too inbred to show any evolutionary potential (Fig. 13.2).

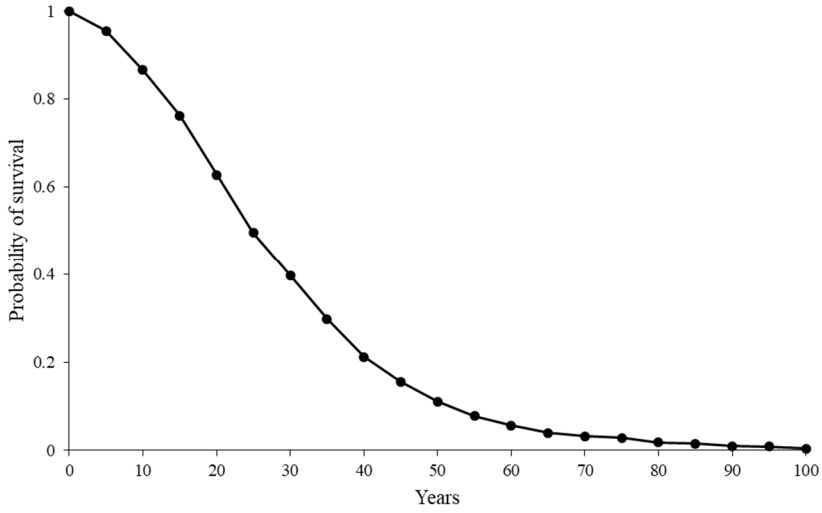


Figure 13.1. Probability of survival. Extracted results of the PVA ran for 1000 iteration over 100 years for *Dryophytes suweonensis* in the Republic of Korea. The probability of extinction of the species after 100 years is > 0.99.

Across all years, the mean growth rate (r) was - 0.26 (SD = 1.81). Populations with the lowest population size (Jincheon, Suweon, Gimpo and Haseong) saw their median time of first extinction < 2 years, while the largest populations, Asan, Iksan and Paju, saw their median time of first extinction 14, 11 and 8 years from now respectively. The average time to extinction is notably slightly higher (Fig. 13.3).

DISCUSSION

Our analysis shows that *Dryophytes suweonensis* may go extinct within 10 years, and the probability of extinction over 100 years is 1. However, (1) this is only if nothing is done, which will hopefully not be the case, despite this statement potentially negative to the "credibility" of the research (Blockstein 2002; Lackey 2007; Chan 2008; Parsons 2013). However, famous conservationists and founders of conservation biology suggest that the projection of numbers by scientists is a requirement to ensure the survival of a population in the field (Soulé 1986; Soulé 1987; Boyce 1992). (2) It only is a probabilistic model, and not deterministic. Besides, many other factors need to be added and corrected based on the future knowledge acquisition, and conservation efforts may also one day have to be plugged in.

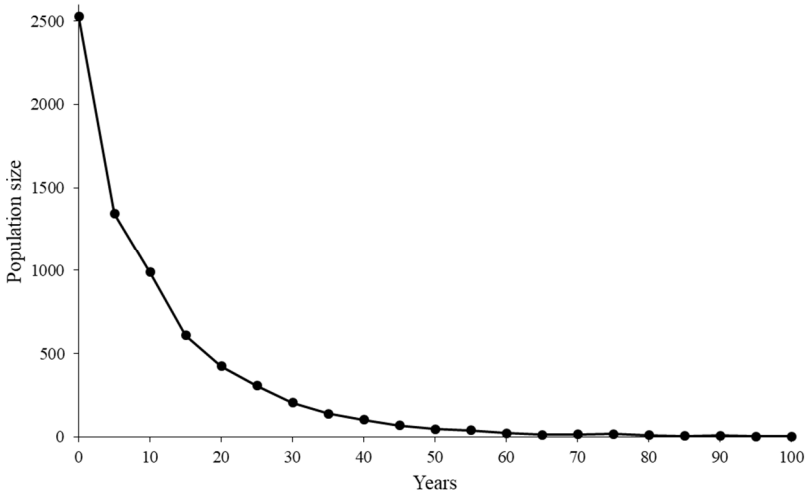


Figure 13.2. Population size. Extracted results of the PVA ran for 1000 iteration over 100 years for *Dryophytes suweonensis* in the Republic of Korea. The population size of the species after 100 years is < 5 (SD = 70.55).

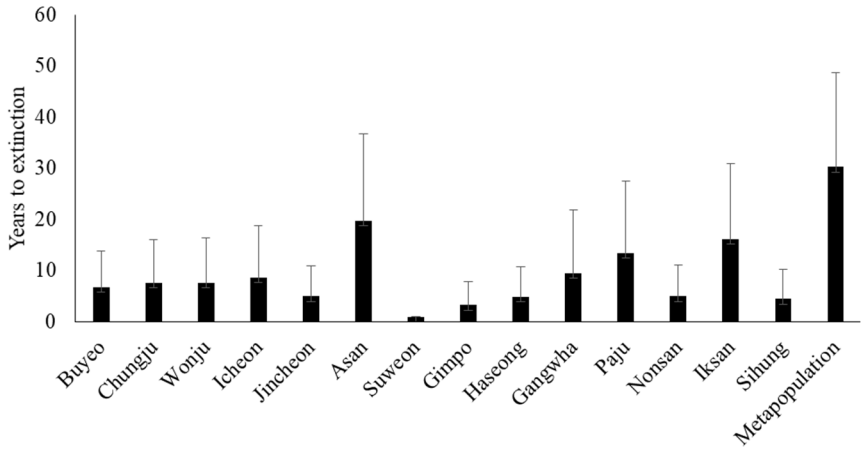


Figure 13.3. Average time to extinction per population size. This value is higher than the median time to extinction provided in the text. Vertical whiskers are standard errors. Extracted results of the PVA ran for 1000 iteration over 100 years for *Dryophytes suweonensis* in the Republic of Korea.

The loss of *D. suweonensis* may not affect the day to day life of people living in the same environment, or on the vicinities of its distribution. However, *D. suweonensis* is an evolutionary significant species (Dufresnes et al. 2015), and if the species is loss, it will contributes to the impoverishment of the biodiversity, and the "empty forest syndrome" will be transferable to the night soundscapes of rice paddies.

There is also another potential development for the species as it is present in the Democratic People's Republic of Korea (Chun et al. 2012), with populations as far North as the localities directly south of the border with China (Author's unpublished data). The status of these population is so far unknown, but as long as they are not extirpated, potential translocation to maintain genetic diversity is an option (see Wright et al. 2014 for instance). Another point to consider is the potential synonymy with *D. immaculatus* (Dufresnes et al. 2016; Borzée *Chapter 10*), although uncertain so far, which could provide a large reservoir population of the species in China. However, this would raise other questions as there is no population connectivity around the yellow sea and the two clades may prove to be different conservation units.

General conclusion

Through this work, I first demonstrated that the Suweon Treefrog (*Dryophytes suweonensis*) is present on a wider range than originally known. I also highlighted that the species is now breeding in rice paddies only, with the last semi-natural site converted into a gold field in 2015, and that *D. suweonensis* does not occur within a single protected area. Interestingly, the current area of occupancy of the species includes large stretches of lands reclaimed on tidal flats, while some areas flooded with specific water bodies are exempt of individuals. This may relate to the species sensitivity to water pollution, as it is less likely to be present at sites with high concentrations of pesticides and fertilisers. When focusing on the spatial distribution of *D. suweonensis*, it was clear that the range of the species was highly fragmented, with 14 isolated populations. The population size was positively correlated with connectivity of localities within the population through branches less than two km long and the presence of rice paddies between localities. The population size was negatively correlated with the presence of roads between localities.

When focusing on a smaller scale but over several years, the number of calling *D. suweonensis* was influenced by environmental and abiotic variables. Encroachment was an important factor defining both the number of calling individuals and the variation in the number of calling individuals between years. There was a large variation in the number of

calling individuals at some localities between years, although the majority of sites displayed minimal variations. Finally, when correcting for the average variation per year, I found a general trend of decreasing population size, and an important decrease in the number of calling individuals at the northern localities of the range.

Despite its smaller size, *D. suweonensis* displayed a significantly larger mass for locomotor muscles, but a longer delay for responses to stimuli and a lower endurance than *D. japonicus*. Furthermore, *D. suweonensis* was in average, but non-significantly, less exploratory but faster than *D. japonicus*. The combined lower cognitive delay for behavioural responses of *D. suweonensis* and the better endurance of *D. japonicus* make the later species more fit to its environment, allowing for faster escape and wider dispersal range. However, both species are present in the same habitat, and tracking movement patterns of males from both species revealed spatial and temporal differentiation in microhabitat use for calling and resting during the breeding season. Males of both *D. suweonensis* and *D. japonicus* are present in the same microhabitats, and both species are calling from rice paddies and resting in grass and bush. However *D. suweonensis* moved into rice paddies and produced advertisement calls three hours earlier than *D. japonicus*, and was subsequently present earlier at all microhabitats. When focusing on rice paddy use, the two species were isolated, with *D. japonicus* on the edges

and *D. suweonensis* in the interior. Male *D. suweonensis* moved towards the edges of rice paddies when male *D. japonicus* were removed from the area, whereas male *D. japonicus* hardly moved when male *D. suweonensis* were removed. Thus, there is asymmetric interspecific competition between the two species, and the calling locations of *D. suweonensis* are affected by the calling activity of *D. japonicus*.

The next set of results demonstrated that the rice cultivation phase was the most important factor to predict the seasonality for the calling activities of both species. The peak calling activities of both treefrog species matched with the optimal hydroperiod in the rice paddy, although *D. japonicus* started breeding earlier than *D. suweonensis*. Besides, *D. japonicus* breeding behaviour was influenced by environmental variables such as temperature, whereas *D. suweonensis* seems to require the plantation of rice seedlings to initiate breeding activities. Indeed, breeding activity of *D. suweonensis* seemed to be triggered by flooding of rice paddies for agricultural purposes.

The wintering ecology of the two *Dryophytes* species was also found to be different, with *D. japonicus* found to be migrating towards forests for over-wintering, and back to rice paddies for breeding in spring. By contrast, *D. suweonensis* was found to hibernate buried in the vicinity of rice paddies, its breeding habitat. This difference in behaviour highlights different ecological requirements between the two species, and

may result from the segregated evolutionary history.

While the genetic diversity at a locality was significantly related to the sphericity of the site boundaries, the genetic analyses revealed fewer haplotypes for *D. suweonensis* than for *D. japonicus*. Besides, there was a generalised, bi-directional and geographically widespread hybridisation between the two species. Evidence of fertile back-crosses was provided by relatively high numbers of individuals in cytonuclear disequilibrium, and the presence of hybrid individuals twice the distance of species' distribution-width away from the distribution limit. This highlights the threat of extinction through hybridisation weighting on *D. suweonensis*. Furthermore, when broadening to the context of North East Asian *Dryophytes*, there was a continuous genetic variation through haplotypes of *D. suweonensis* and *D. immaculatus* forming a ring around the Yellow Sea. This makes difficult to conclude on either a ring-species or a ring of species around the shallow Yellow Sea, but I recommend the use of other cues such as call characteristics and morphology to determine the species or sub-species status of these two clades.

When estimating the future of *D. suweonensis* through a population viability analysis, the results showed that as of 2017, the population will drop below 1000 individuals within nine years, below 500 within 20 years, and the species' probability of extinction was 1.00 within 100 years. Thus, the translocation pilot project to help the species avoid

extinction showed that translocation of metamorphs can be successful, but management coordination is a conditional requirement. Following the translocation in 2015, the population was monitored in 2016, when calling male *D. suweonensis*, tadpoles and juveniles were identified. Juveniles were seen until the last week of 2016 before putative hibernation. However, a single male was recorded calling in 2017. The population was consequently considered functionally extinct.

All the work conducted here concludes on the multiple threats exercising pressure on the Suweon Treefrog, ranging from encroachment, fragmentation, loss of natural habitat, behavioural out-competing, ecological niche restriction and potential character displacement because of competition, temporal restriction to breeding activities because of rice paddy management, threat related to microhabitat use for hibernation, hybridisation and water pollution. These result in the decreasing of population size on long term, with potential extinction within decades. The positive point is that translocation of the species to adequate habitats for its conservation can work in a properly managed framework.

초록

현재 일어나고 있는 여섯번째 대멸종의 선두 주자는 바로 양서류이다. 예측불가능할 정도로 많은 수의 양서류가 멸종의 위기에 처해있다. 양서류 개체군은 1) 서식지 파괴, 2) 화학 비료와 화학 오염, 3) 중파장 방사선, 4) 질병, 5) 외래종, 6) 개발, 7) 기후변화, 그리고 8) 앞에 서술된 여러가지 요인들의 혼합된 효과등을 이유로 급격하게 감소하고 있다. 어떤 종에게는 다른 요인들 보다 특정 요인들이 더욱 더 중요하게 작용할 수 있다. 도시 개발과 자연 서식지 침식은 거의 모든 멸종위기종의 주요 위협요인이다. 신석기 농업 혁명 이래로 인류는 대다수 양서류의 주된 번식지이기도 한 저지대에 터를 잡고 살기 시작했다. 이 경향은 현재까지도 계속되어, 전세계의 대도시들을 이루게 되었다. 도시 개발은 메타개체군 간의 연결성 역시 감소시켰으며, 이는 유전적 다양성의 감소로 연결되었다. 경관의 인위적인 변화는 종의 행동과 생태에 영향을 미쳐, 새로운 경쟁 역학을 만들어냈다. 예를 들어, 인간의 농경 활동과 자연 서식지의 사라짐으로 인하여 지리적으로 분리되어 있던 종들이 접촉하게 되고, 이는 유전자 이입으로 인한 멸종으로 이어질 가능성이 있다. 또한, 화학 약품의 사용과 외래종의 도입은 이미 위태롭고 약해진 종에게 큰 타격이 될 수 있다.

본 학위 논문의 목표는멸종위기종인 수원청개구리(*Dryophytes suweonensis*) 의 현재 상황을 평가하고, 이를 통하여 궁극적으로 종의 생존 가능성을 예측하는 것이다. 이 종은 한반도 서부 지방의 좁은 범위에 서식하며, 논에서 일생을 보낸다. 또한 수원청개구리는 광범위한 서식지를 가진 청개구리(*D. japonicus*) 와 함께 발견되며, 이 두 종은 폭 넓은 교류를 가진다. 본 학위 논문의 가장 첫 단계는 수원청개구리의 한반도 내 전 서식지를 추정하는 것이었다. 그 후, 종 분포와 개체군 크기 데이터를 사용하여 종의 메타개체군 연결성과, 서식지 크기와 적합성(fitness)의 관계를 추정하였다. 그리고 경쟁과 먹이원 중첩, 청개구리와의 서식지 분리 또한 추정하였다. 두 청개구리종의 공간적 분포와 인위적인 요인으로 인해 변형된 번식 전략을 연유로, *D. suweonensis* 와*D. japonicus*의 교배종과, 유전자 이입으로 인한*D. suweonensis*의 멸종 가능성에 대하여 조사하였다. 또한, 근래의 진화 역사를 알아보기 위하여, *D. suweonensis*와 가까운 관계에 있는 동북아시아의Hylid 종의 계통적 관계에 대해 연구하였다. 그 후, 종의 겨울 생태와 농경 활동으로 인한 화학 약품 유출에 관련된 위협요인들을 추정하였다. 마지막으로, 시험적 연구를 통하여 종의 이주(translocation)과 재도입 가능성을 추정하였다. 또한 *D. suweonensis*의 멸종

가능성을 평가하기 위하여 개체군 실용성 분석을 돌리기 전에, 장기적인 개체군 동향을 확립하였다.

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Appendix

Annex 5.1. Several types of antenna (whole length, length from the diode to the bend and general shape) were empirically investigated to find the optimal combination for the best range. It was shown that one leg is less efficient than two legs for the antenna (Range Variation (RV) = 14%). The length from the diode to the bend provides a better range at 1 cm versus 0.2 cm (RV = 26%). The bending of the two legs at fifteen degrees after the diode versus parallel legs also yields a better range (RV = 57%). The range is also a function of the length of the legs of the antenna, which were adapted to the weight of the individual, independently for each treefrog.

Legs	Leg size	Loop size	Bent	Range
2	15 cm	1 cm	0°	8.4 m
1	8 cm	0.2 cm	0 °	3.6 m
1	8 cm	1 cm	0 °	9.6 m
2	8 cm	1 cm	0 °	13.8 m
2	8 cm	1 cm	15 °	24.3 m

Annex 9.1. Description of the mtDNA and microsatellite markers tested for cross amplification in *Dryophytes japonicus* and *D. suweonensis*. The mtDNA CO1 marker is on top of the table. The six microsatellite primer pairs for which cross-amplification was successful are in bold. The mtDNA primers originate from Hebert et al. (2004) while primers for the nuclear genes are from Bossuyt & Milinkovitch (2000), Bossuyt & Milinkovitch (2000), Bonacum et al. (2001), Crawford (2003), Wiens et al. (2005), Smith et al. (2005), and Faivovich et al. (2005). In bold are the primer pairs that cross-amplified for the two species.

Origin DNA	Gene name	Primer name	Primer sequence (5'-3')
mtDNA	CO1	**LEPF1**	ATTCAACCAATCATAAAGATAT'
		LEPR1	TAAACTTCTGGATGTCCAAAAA
nuDNA	POMC	**POMC-1**	GAATGTATYAAAGMMTGCAAGATGGWCCT
		POMC-2	TAYTGRCCCTTYTTGTGGGCRTT
nuDNA	POMC	POMC-6	TCTGCMGAGTCACCRGTGTTTC
		POMC-7	TGGCATTTTTGAAAAGAGTCAT
nuDNA	c-myc	c-myc 1U	GAGGACATCTGGAARAARTT
		c-myc ex2dR	TCATTCAATGGGTAAGGGAAGACC
nuDNA	c-myc	**c-myc ex3F2**	AYGTNCCYATYCAYCAGCACAACT
		c-myc ex3R3	TCKCGNAKGAGYCKYCGCTCRTC
nuDNA	c-myc	**c-myc 3L**	GTCTTCCTCTTGTCRTTCTCYTC
		c-myc ex3F	CCCACCAGTCCAGACCTCACCACAG
nuDNA	RAG1	**R1-GFF**	GAGAAGTCTACAAAAAVGGCAAAG
		R1-GFR	GAAGCGCCTGAACAGTTTATTAC
nuDNA	TYR	Tyr 1C	GGCAGAGGAWCRTGCCAAGATGT
		Tyr 1G	TGCTGGGCRTCTCTCCARTCCCA
nuDNA	SIAH	**Sia1**	TCGAGTGCCCCGTGTGYTTYGAYTA
		Sia2	GAAGTGGAAGCCGAAGCAGSWYTGCATCAT
nuDNA	RHO	**Rhod1A**	ACCATGAACGGAACAGAAGGYCC
		Rhod1C	CCAAGGGTAGCGAAGAARCCTTC

Annex 9.2. List of the six primer pairs used for genotyping together with the PCR volumes and protocols. The PCR reactions were in a total volume of 20 µL, completed with PCR grade water. Volumes are in µl. Primers concentration is 10 µM.

Primer pair	POMC	c-myc 2	c-myc 3	RAG1	SIAH	RHO
DNA template	1	1	1	1	1	1
Buffer 10X	2	2	2	2	2	2
dNTP	1	1	1	1	1	1
Primer (F)	0.9	0.9	0.9	0.9	0.9	0.9
Primer (R)	0.9	0.9	0.9	0.9	0.9	0.9
Takara Taq	0.2	0.2	0.2	0.2	0.2	0.2
Annealing Temp	47	47	50	47	47	47
Fluorescent dye	FAM	HEX	FAM	FAM	HEX	HEX

Annex 9.3. Distribution of the microsatellites alleles sizes for *Dryophytes suweonensis* (Ds) and *D. japonicus* (Dj).

Cmyc2		Cmyc3		Pomc		Rag1		Rho		Siah	
Dj	*Ds*	*Dj*	*Ds*	*Dj*	*Ds*	*Dj*	*Ds*	*Dj*	*Ds*	*Dj*	*Ds*
335	335	220	220	633		147		144		166	166
336	336	221				156	156	166	166	167	167
337		222	222	634		157	157	167	167	445	
338	338	223	223		636	158		357		446	
339	339	224	224	638			162	358	358	447	
340	340	225	225	639	639	164		359	359	448	448
341	341	226	226	640	640	165	165	360	360	449	449
342	342	344	227	641	641		175	361	361	450	450
343	343	345	228	642	642		190	362	362	451	451
344	344		233	643	643	201		363	363	453	453
345	345		344	645		202			365		
346	346		405	646	646	205	205		366		
347	347				647	209					
348	348			648	648		213				
349	349				649		224				
350	350				650	229					
351	351						238				
352	352					239					
353	353					240					
354	354					242	242				
355	355					243	243				
356	356					244	244				
	357					245	245				
	358					246	246				
	359					247	247				
	360					248	248				

Annex 10.1. Specimen information used in this study. For each specimen, species identity, GenBank accession numbers for each partition, specimens with voucher code (if available), and locality are included. *indicates an update from *Hyla* to *Dryophytes* (Duellman et al., 2016), independently of the name present on the GenBank upload. ROK stands for Republic of Korea.

Taxon	GenBank No.		Specimen voucher	Locality
	12S		16S	no./ isolate no.
Hyla arborea	DQ055835	DQ055814	-	Croatia, Donja Korita
Hyla chinensis	KP742571	KP742700	IOZCAS4796	China: Zhaowu, Fujian
*Dryophytes femoralis**	DQ055838	DQ055819	MVZ137344	USA: Emanuel, Georgia
*Dryophytes immaculatus**	KP742584	KP742712	CIBLJT060811	China: Conghua, Guangdong
	KP742585	KP742713	CIBLJT060812	China: Conghua, Guangdong
	KP742586	KP742714	SCUM0606003	China: Jingzhou, Hubei
	KP742587	KP742715	SCUM0606004	China: Jingzhou, Hubei
	KP742588	KP742716	SZ100604	China: Sangzhi, Hunan
	KP742589	KP742717	CIBLJT070515	China: Conghua, Guangdong
*Dryophytes japonicus**	KP742597	KP742724	CIBLJT070602	China: Hailin, Heilongjiang
	KP742598	KP742725	CIBLJT070607	China: Shenyang, Liaoning
	KP742599	KP742726	ZISPRussia101001	Russia: Ussuriysk Distr.
	KP742600	KP742727	ZISPRussia101002	Russia: Ussuriysk Distr.
*Dryophytes suweonensis**	1	1	BHV383	Geumchon, ROK
	2	2	BHV387	Geumchon, ROK
	3	3	BHV390	Geumchon, ROK
	4	4	BHV391	Geumchon, ROK
	5	5	BHV393	Geumchon, ROK
	6	6	BHV394	Geumchon, ROK
	7	7	BHV396	Geumchon, ROK
	8	8	BHV397	Geumchon, ROK
	9	9	BHV398	Geumchon, ROK
	10	10	BHV399	Geumchon, ROK

	11	11	BHV400	Geumchon, ROK
	12	12	BHV403	Geumchon, ROK
	13	13	SUR12	Geumchon, ROK
	14	14	SUR32	Cheonan, ROK
	15	15	SUR33	Cheonan, ROK
	16	16	SUR34	Cheonan, ROK
	17	17	SUR35	Cheonan, ROK
	18	18	SUR36	Cheonan, ROK
	19	19	SUR38	Cheonan, ROK
	20	20	SUR39	Cheonan, ROK
	21	21	SUR40	Cheonan, ROK
	22	22	SUR43	Cheonan, ROK
	23	23	LTR1	Iksan, ROK
	24	24	LTR2	Iksan, ROK
	25	25	LTR3	Iksan, ROK
	26	26	LTR4	Iksan, ROK
	27	27	LTR5	Iksan, ROK
	28	28	LTR6	Iksan, ROK
	29	29	LTR8	Iksan, ROK
	30	30	LTR9	Iksan, ROK
	31	31	LTR10	Iksan, ROK
	32	32	LTR11	Iksan, ROK
Smilisca fodiens	AY819387	AY819519	MVZ132994	Mexico: Sonora
Pseudacris nigrita	AY819386	AY819518	MVZ145454	USA: North Carolina